



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Paulson *et al.*

Application No.: 10/007,331

Filed: November 9, 2001

For: PRACTICAL *IN VITRO*
SIALYLATION OF RECOMBINANT
GLYCOPROTEINS

Examiner: Prouty, Rebecca E

Art Unit: 1652

DECLARATION OF
DAVID A. ZOPF, M.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, David A. Zopf, M.D. declare as follows:

1. I am currently Executive Vice President and Chief Scientific Officer at Neose Technologies, Inc. (hereinafter "Neose"). My qualifications are discussed in my declaration submitted with the response mailed May 18, 2004 (hereinafter my "First Declaration"). My current *Curriculum Vitae* is attached to my First Declaration.

2. In my First Declaration, I provided evidence that the claimed technology has enjoyed commercial success. In particular, I noted that at the time my first declaration was signed (October 16, 2001) more than 20 successful feasibility studies had been completed. In addition, I provided evidence of ongoing collaborative research and development agreements with other companies to develop commercial manufacturing methods. The response mailed May 18, 2004 provided additional evidence of successful collaborations since my first declaration was signed.

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3. In the present Office Action, I understand that the Examiner has questioned whether the demonstrated commercial success is related to the claimed invention, despite my statement to that effect in Paragraph 11 of my first declaration. This declaration is submitted to provided further evidence that the commercial success is a result of the merits of the present invention and not related to other factors.

4. As explained in the patent application, achieving and maintaining proper glycosylation is a major challenge in biotechnology manufacturing, and one that affects the industry's overall ability to maximize the clinical and commercial gains possible with these agents. Utilizing cell systems to produce recombinant glycoproteins requires balancing the cells' ability to produce the protein with their ability to attach the appropriate carbohydrates.

5. Attached is a copy of a paper (Zopf and Vergis, *Pharmaceutical Visions*, pp10-14, Spring 2002, Exhibit 1) that sets out some of the technical problems encountered in recombinant production of glycoproteins. As explained at page 11 and illustrated in Figure 3, none of the currently used recombinant expression systems produce proteins having glycosylation as found in human cells.

6. The problems in glycosylation result in low yields of usable product, which contribute to the cost and complexity of producing these drugs. For example on page 11 of Zopf and Vergis, we note that underglycosylation results in manufacturing losses of up to 80% of the product. Incorrect sialylation, in particular, affects the half-life of the drug, resulting in the need to administer higher and more frequent doses. This affects the cost of therapy, and potentially, the incidence of side effects. Thus, the ability to manufacture therapeutic glycoproteins with improved glycosylation has been an important strategic goal of pharmaceutical and biotechnology companies.

7. The methods of the present invention are very effective in addressing this long felt need. The claimed methods require that the step of contacting a glycoprotein with a sialyltransferase and other reactants occurs *in vitro*. As shown in the

specification and in the attached exhibits, the method result in sialylation of at least 80% of the terminal galactose residues on the glycoprotein. The high degree of sialylation achieved using the methods of the invention is also shown in Figure 3 of the Zopf and Vergis. These two limitations (*in vitro* reaction and high degree of sialylation) are directly related to the success of the claimed process.

8. In our experience, producers of recombinant therapeutic proteins have been interested in the claimed *in vitro* methods because the methods can be used to improve glycosylation without altering the host cells or culture conditions that have been optimized for other purposes, such as yield. In addition, the methods can be used in combination with in any expression system, such as bacteria, yeast, fungi, or plants. Prior to the advent of the claimed technology, producers of recombinant therapeutic glycoproteins had no commercially feasible means for achieving this goal.

9. The commercial success of the invention has been amply demonstrated by my First Declaration and the additional evidence provided with the last response. Moreover, the evidence of record shows the high degree of sialylation and the resulting improvement in biological properties of glycoproteins treated according to the claimed methods. These improvements have been publicly noted by our collaborators, thus demonstrating that improvement are generally recognized by those of skill in the art, not just by those at Neose.

10. For example, Exhibit 2¹ is a copy of a presentation made by employees of Eli Lilly at the Biotechnology Industry Organization Meeting (BIO) held on June 26, 2001, in San Diego, California. As described therein, a "glycoprotein X" (GPX) was sent by scientists at Eli Lilly to Neose for resialylation. The presenters conclude in the last slide of the presentation by noting that the methods restored sialic acid on 99% of exposed Gal residues and N-linked glycans. These improvements, the Eli Lilly scientists concluded, resulted in slower plasma clearance and an increase in steady-state plasma concentration.

11. Exhibit 3 is copy of Thomas *et al.* *Glycobiology* 14:883-93 (2004), which is the same as the manuscript provided as Appendix 1 to the response mailed May

¹ This document is identical to Exhibit 3 to my First Declaration.

18, 2004. This publication describes the results of research carried out by Neose scientists in collaboration with scientists from Avant Immunotherapeutics. As noted earlier, the glycosylation reactions described there (both sialylation and fucosylation) resulted in nearly all of the carbohydrates of the target protein terminating in the desired oligosaccharide structure (termed sLe^x). The proteins therefore had a ten-fold increase in affinity for the target receptor (termed E-selectin). The authors, including Avant Immunotherapeutics scientists, concluded that the *in vitro* glycosylation of the invention "reduces heterogeneity of the glycan profile, improves pharmacokinetics and enhances carbohydrate mediated binding to E-selection." (see Abstract). Thus, both Avant Immunotherapeutics and the reviewers of this manuscript felt that the observed improvements in binding of the modified protein was commercially valuable and worthy of publication in a respected scientific journal.

12. In conclusion, the claimed methods have been extremely effective in restoring high degree of sialylation. Indeed, the degree of sialylation achieved is routinely well over 80%. This has resulted in improved biological function. Moreover, the improved results are publicly noted by our collaborators and are accepted by third party reviewers at respected journals. These results coupled with the fact that potential partners need not modify existing culture conditions have been directly responsible for the commercial success of the claimed invention.

13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: November 23, 2004



David A. Zopf, M.D.

Glycosylation: a critical issue in protein development and manufacturing

David Zopf and George Vergis review the applications of in vitro glycoprotein remodelling, which utilises glycosyltransferases to attach the appropriate carbohydrates to the protein after it is expressed and secreted by the host cell. They argue that this new technology has the potential to help the industry solve its protein production issues

Recombinant proteins and monoclonal antibodies offer great promise as therapeutics for a myriad of diseases. There are more than 360 biotechnology drugs in development for over 200 different conditions¹ (Figure 1). Many of these drugs are glycoproteins – proteins that include complex carbohydrates as part of their structure. The process by which these carbohydrates are attached to proteins is called glycosylation. Glycosylation patterns are important to the structure and function of glycoproteins. Achieving and maintaining proper glycosylation is a major challenge in biotechnology manufacturing, and one that affects the industry's overall ability to maximise the clinical and commercial gains possible with these agents.

Most recombinant therapeutic glycoproteins, including the well-known drugs Avonex (interferon beta 1-a, Biogen, Inc) and EPOGEN/EPREX (epoetin alfa, Amgen Inc/Ortho Biotech), are produced in living cells – Chinese hamster ovary (CHO) cells – in an attempt to correctly match the glycosylation patterns found in the natural human form of the protein and achieve optimal in vivo functionality. However, utilising cell systems to produce glycoproteins requires balancing the cells' ability to produce the protein with their ability to attach the appropriate carbohydrates. CHO cells engineered to produce large

FIGURE 1: BIOTECHNOLOGY MEDICINES IN DEVELOPMENT BY THERAPEUTIC CATEGORY	
AIDS/HIV infection/related disorders	19
Autoimmune disorders	19
Blood disorders	9
Cancer/related conditions	175
Diabetes-related conditions	7
Digestive disorders	11
Eye conditions	3
Genetic disorders	11
Growth disorders	3
Heart disease	26
Infectious diseases	39
Infertility	5
Neurological disorders	28
Respiratory diseases	22
Skin disorders	1
Transplantation	13
Other	29

Note: some medicines are listed in more than one category

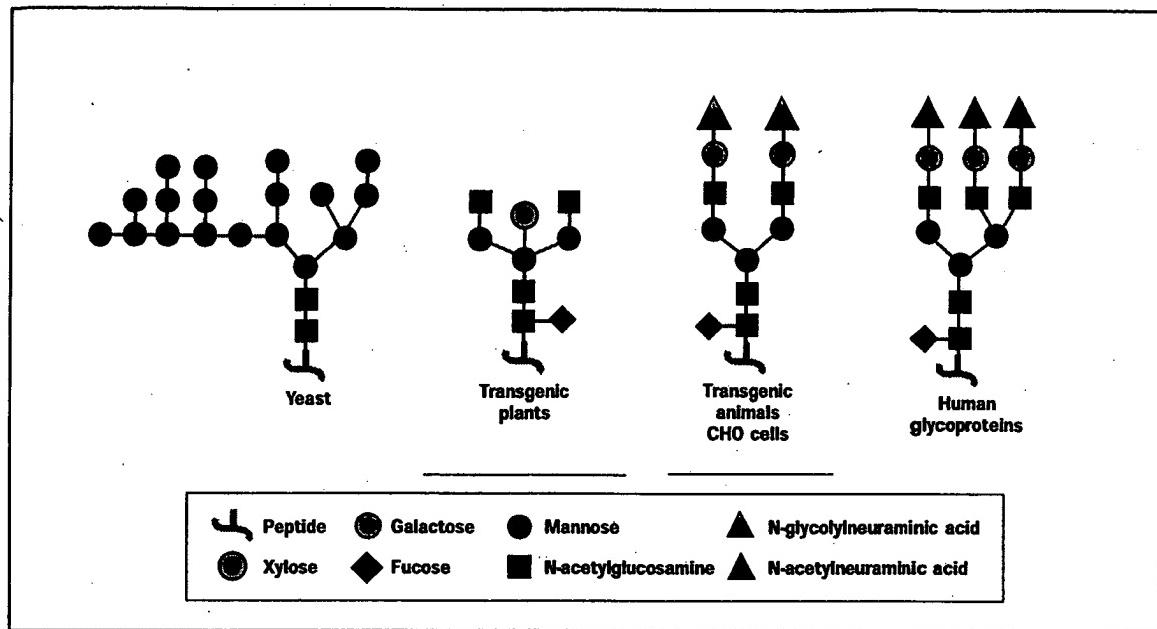
Source: *New medicines in development: biotechnology* Pharmaceutical Research and Manufacturers Association

quantities of a specific protein often do not maintain the proper level of glycosylation. This results in low yields of usable product, which contributes to the cost and complexity of producing these drugs. Incorrect glycosylation also affects the

half-life of the drug, resulting in the need to administer higher and more frequent doses. This affects the cost of therapy, and potentially, the incidence of side effects. Low yields are a significant contributor to the critical worldwide shortage of biotechnology manufacturing capacity. A recent report by David Molowa, a biotechnology equity research analyst, predicted that demand for manufacturing capacity will exceed current capacity by a factor of four by 2005². Thus, the ability to manufacture these drugs is becoming an important strategic asset of pharmaceutical and biotechnology companies.

Because of these issues, the pharmaceutical industry continues to search for better ways to manufacture glycoproteins. Alternative expression systems, such as transgenic animals and plants, have received industry and media attention because they may be able to significantly increase product yields at lower cost. However, achieving the correct glycosylation patterns remains a problem with these systems and is a significant barrier to their widespread adoption for manufacturing proteins for parenteral use. A new technology, in vitro glycoprotein remodelling, is available that utilises specific enzymes, glycosyltransferases, to attach the appropriate carbohydrates to the protein after it is expressed and secreted by the host cell. Employing this technology to ensure correct glycosylation of proteins has the

Figure 2.
Comparison of glycosylation among alternate expression systems



potential to help the industry solve its protein production issues.

GLYCOSYLATION - A PRIMER

Glycoproteins consist of oligosaccharides, or sugar chains, covalently linked to proteins. The predominant sugars found on human glycoproteins include galactose, mannose, fucose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), and N-acetylneuraminic acid (the human form of sialic acid). Correctly attaching these sugars through the process of glycosylation is the most extensive post-translational modification made to proteins by eukaryotic cells. There are two types of glycosylation. N-glycosylation begins with the linking of GlcNAc to the amide group of asparagine. O-glycosylation most commonly links GalNAc to the hydroxyl group of serine or threonine. The processing of N-glycans occurs cotranslationally in the lumen of the endoplasmic reticulum and continues in the Golgi apparatus for N-linked glycoproteins. O-linked glycosylation occurs post-translationally in the Golgi apparatus. The attachment of sugars is catalysed by specific glycosyltransferases. Many of these enzymes are extremely sensitive to stimuli within the cell in which the glycoprotein is expressed. As a result, the specific sugars attached to an individual protein depend on the cell type in which

the glycoprotein is expressed and on the physiological status of the cell.

The carbohydrate components of glycoproteins affect the functionality of the molecule by determining protein folding, oligomer assembly and secretion processes. Without the proper shape, the ability of the protein to interact correctly with its receptor is affected, possibly affecting function. Glycosylation may affect solubility and prevent aggregation. Carbohydrates also affect the rate of clearance of glycoproteins from the bloodstream. N-glycans on human glycoproteins have a specific order terminating in N-acetylneuraminic acid (sialic acid). Missing terminal sialic acids on a glycoprotein expose underlying galactose residues, which are a signal to the liver to remove the glycoprotein from circulation.

RECOMBINANT PROTEINS

Recombinant proteins and monoclonal antibodies require a host organism for expression of the desired protein. Although protein expression systems produce correct amino acid sequences, the glycosylation remains that of the host (Figure 2). In addition, these systems do not maintain complete glycosylation under high-volume production conditions.

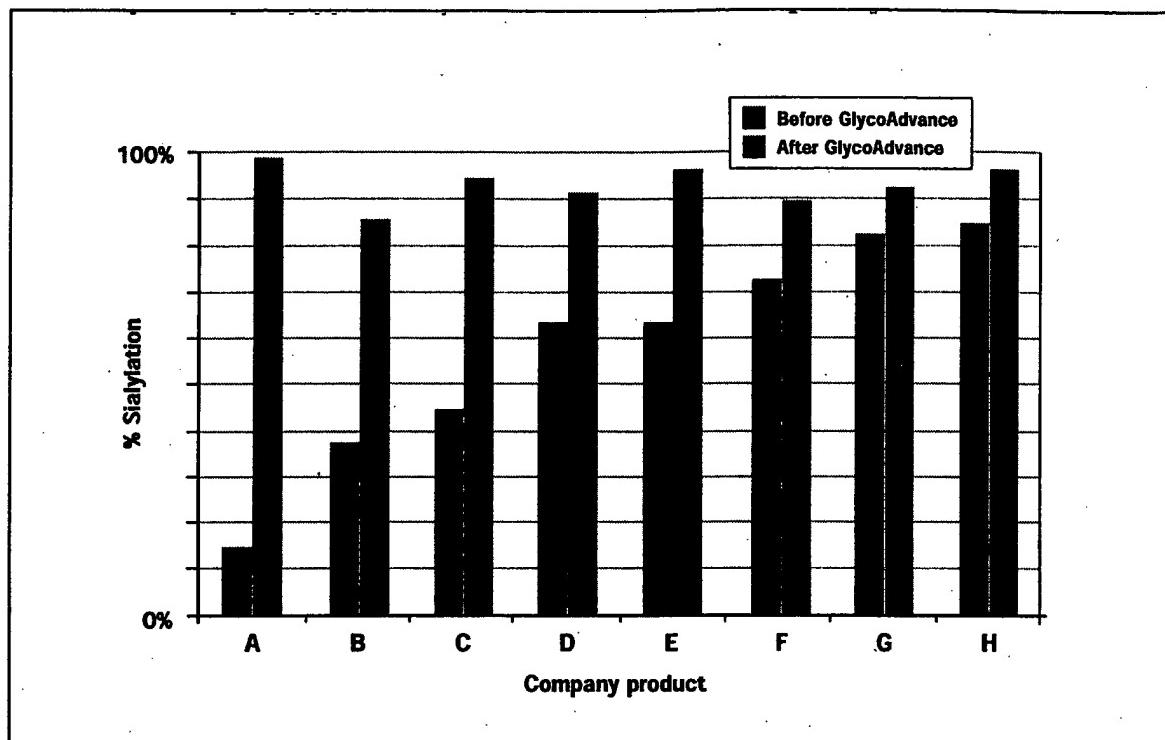
Common bacterial expression systems such as *Escherichia coli* do not

glycosylate recombinant mammalian proteins.³ Yeast and fungal expression systems offer a simple production process with high yield, powerful secretory pathways, and some limited post-translational modifications. However, the glycosylation patterns are significantly different from what occurs in mammalian cells. Yeast cells add high-mannose structures that are non-human. Although these structures may not be immunogenic, the expressed glycoprotein will have a short half-life. There is also evidence to suggest that different O-glycosylation patterns occur in yeast cells and mammalian cells.⁴ Insect cells put on shorter mannose structures than yeast. Again, while not likely immunogenic, these foreign patterns affect overall half-life of the recombinant protein.

The published studies on the production of human proteins in plants have suggested that plants often add simple N-glycan structures that lack galactose and terminal sialic acids, which will compromise activity. Another obstacle may be the presence of alpha 1-3 linked core fucose and xylose residues that are potentially immunogenic.^{5,6}

CHO cells, the system most commonly used today for recombinant protein manufacturing, glycosylate close to human but do not maintain complete glycosylation under production conditions.

Figure 3.
GlycoAdvance has consistently improved glycosylation



Transgenic animals are being studied as an alternative to traditional CHO cell production processes. Transgenic animals provide a potentially less expensive source of production for proteins compared to traditional cell culture systems. In recent years, the number of production systems has increased, with more than 20 companies worldwide involved in pharmaceutical protein production in various transgenic animals. The protein, antithrombin III, produced in the milk of transgenic goats, is currently in phase III clinical trials.

While transgenic expression systems may solve the problems of protein production yields and may lower cost, they do not solve the problem of protein glycosylation. As with CHO cells, cells of the lactating mammary gland may underglycosylate glycoproteins they secrete. A potential concern is that most transgenic systems put on a non-human form of sialic acid, N-glycolyneuraminic acid. Whether or not this is a problem may become evident as high-dose, chronic-use protein therapeutics become more widely used.

A review of interferon gamma, a recombinant protein that has been expressed in three different systems, offers insight into the types of glycosylation differences that occur among expres-

sion systems. Interferon gamma produced in CHO cells has substitutions with a fucose residue, and high mannose oligosaccharide chains. Interferon gamma produced in transgenic mice shows considerable site-specific variation in N-glycan structures. Interferon gamma produced from insect cell culture is associated with tri-mannosyl core structures. These differences highlight the importance of monitoring glycosylation patterns and noting the effect of variances in glycosylation on the structure and function of the recombinant protein.

COMPETITIVE ADVANTAGE

As an example of the critical link between correct glycosylation, optimal function and competitive advantage, we examine recombinant human erythropoietin. Recombinant erythropoietin has worldwide sales of nearly \$5 billion annually. The natural hormone is a 165 amino acid glycoprotein with one O-linked and three N-linked glycosylation sites. The effects of correctly matching these glycosylation patterns in the recombinant product are complex. Although unglycosylated *E. coli*-derived erythropoietin exhibits full biological activity *in vitro*, it has very low activity in animal studies.¹⁴ Desialylated erythropoietin shows enhanced activity *in vitro*, but

decreased activity *in vivo*. This is attributed to the binding of galactose residues by hepatocytes in the liver which rapidly bind and clear the glycoprotein.^{10,11} Commercial erythropoietin is produced in CHO cells, and there are considerable manufacturing losses (up to 80%) because underglycosylated material is not used.

A second generation erythropoietin, novel erythropoiesis stimulating protein (NESP, Aranesp, Amgen Inc), was recently approved for treatment of erythropoietin deficiency in patients with chronic renal failure. NESP has five N-linked oligosaccharide chains, compared to the three chains found in recombinant erythropoietin.¹² These extra N-glycans extend the half-life of NESP, with NESP having a half-life three times longer than that of recombinant erythropoietin.¹² The longer half-life permits less frequent dosing of NESP. Patients currently receiving erythropoietin every week will receive NESP every two weeks. Because the drug is administered parenterally, extending the half-life simplifies therapy for both patients and physicians by allowing for less frequent dosing. This may also reduce overall healthcare costs. Most importantly, the less frequent dosing is anticipated to be a significant competitive advantage for Amgen, possibly giving them a piece of the lucrative \$3 billion market

for indications outside of renal failure currently enjoyed by Johnson & Johnson's PROCRIT.

GLYCOPROTEIN REMODELLING

A better understanding of glycoprotein biosynthesis, along with identification and cloning of many of the enzymes involved in this process, has provided a foundation for new technologies that will enable the industry to make optimal use of the discoveries arising from the great strides in genomics. A technology to add missing sugars to carbohydrate chains on a glycoprotein after it has been secreted from the production cell is now available. The proprietary technology is being developed by Neose Technologies, Horsham, Pennsylvania, under the brand name GlycoAdvance. Because this technology decouples protein expression from glycosylation, it has the potential not only to ensure correct and complete glycosylation patterns, but may allow pharmaceutical manufacturers to choose the most efficient production cell lines without having to control for glycosylation.

This technology uses glycosyltransferases to add missing sugar units to the carbohydrate chains via donor sugars known as sugar nucleotides. In a production setting, the GlycoAdvance enzymes and sugar nucleotides are added to the cell culture medium containing the target protein after it has been secreted from the production cell. The step can be easily integrated into existing on-site manufacturing processes. Neose is currently expanding its capacity to manufacture and supply to protein manufacturers the GlycoAdvance enzymes and sugar nucleotides produced under Good Manufacturing Practices for use in large-scale glycoprotein production.

In feasibility studies conducted to date, GlycoAdvance has consistently improved glycosylation (Figure 3, previous page). Studies completed by Neose in collaboration with Biogen and Eli Lilly researchers have indicated that GlycoAdvance technology resulted in nearly complete restoration of the target sugars to the glycoproteins, and GlycoAdvance significantly increased the *in vivo* half-life of the glycoproteins to which it was applied.¹²

LOOKING FORWARD

We are in the midst of explosive growth in the number of biotechnology medicines in development, driven by the rapidly growing number of known drug opportunities emerging from genomics, and the ability to clone and express human proteins. These developments are a major force in the growth of the pharmaceutical and biotech industries. In 2000, worldwide sales of glycoprotein and antibody drugs were about \$16 billion. By 2010, worldwide sales are expected to exceed \$68 billion. Developments in this area are limited by manufacturing production capacities, with capacities lagging far behind demand. These manufacturing limitations are likely to slow the growth in the biotech industry that could be realised if these issues were solved.¹⁴ Industry analysts have estimated that for every \$100m of demand for a drug that goes unfilled, \$1 billion of the drug's market value is destroyed.¹⁵

The bright future of protein therapeutics may be limited only by the ability of the industry to manufacture sufficient quantities of these drugs at a reasonable cost. GlycoAdvance technology offers a solution to the incorrect or incomplete glycosylation associated with both CHO cell and other expression systems. Using GlycoAdvance to add missing sugars to glycoproteins will enable protein manufacturers to correct the problems of reduced product yield, shortened drug half-life and decreased efficacy that result from incomplete glycosylation. ●

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REFERENCES

- ¹Pharmaceutical Research and Manufacturers of America (PhRMA) Survey 2000. *New medicines*

In development biotechnology. Available at <http://www.phrma.org/searchcures/newmeds>.

²Molowa DT. *The State of Biologics* Manufacturing JP Morgan Securities, Inc. 12 March, 2001.

³Letourneau O, Sechi S, Willettebrown J, Robertson MW, Kinet JP. Glycosylation of human truncated Fc-epsilon RI-chain is necessary for efficient folding in the endoplasmic reticulum *J Biol Chem* 1995;270:8249-8256.

⁴Kalsner I, Schneider FJ, Geyer R, Ahorn H, Maurerfog I. Comparison of the carbohydrate moieties of recombinant soluble Fc-epsilon receptor expressed in *Saccharomyces cerevisiae* and chinese hamster ovary cells - different O-glycosylation sites are used by yeast and mammalian cells *Glycoconjugate J* 1992;9:209-216.

⁵Altmann F, Treter V, Kubelka V, Staudacher E, Marz Lecker WM. Fucose in alpha 1,3 linkage to the N-glycan core forms an allergenic epitope that occurs in plant and in insect glycoproteins *Glycoconjugate J* 1993;10:301.

⁶Ma JKC, Hein MB. Immunotherapeutic potential of antibodies produced in plants *Trends Biotechnol* 1995;13:522-527.

⁷Krantz SB. *Erythropoietin Blood* 1991;77:419-434.

⁸Sasaki H, Othner B, Dell B, Fukudam A. Carbohydrate structure of erythropoietin expressed in chinese hamster ovary cells by a human erythropoietin cDNA *J Biol Chem* 1987;262:12059-12076.

⁹Dordal MS, Wang FF, Goldwasser E. The role of carbohydrate in erythropoietin action *Endocrinology* 1985;116:2293-2299.

¹⁰Spivak JL, Hogan BB. The *in vivo* metabolism of recombinant human erythropoietin in the rat *Blood* 1989;73:90-99.

¹¹Goldwasser E, Kung CK, Eliason J. On the mechanism of erythropoietin induced differentiation. The role of sialic acid in erythropoietin action *J Biol Chem* 1974;249:4202-4206.

¹²FDA Approval of Aranesp. Accessed at www.fukumi.co.jp/mm/add/mp_anemia on 28 February, 2002.

¹³Neose News Release 29 June, 2001.

¹⁴Garber K. Biotech industry faces new bottleneck. *Nature Biotechnology* 2001;19:184-185.

¹⁵Andersson R, Mynahan R. The protein production challenge *In Vivo* May 2001;1-5.

Modifying Recombinant Glycoproteins



N Jenkins, E Kattelman, D Witcher
& V Wroblewski

Lilly

Answers That Matter.

EXHIBIT 3

Introduction

- glycoproteins are *rarely* fully glycosylated
- variants arise by incomplete biosynthesis and/or degradation by cellular glycosidases (most frequently sialidase)
- glycoform differences can lead to variable biological activity and differences in pharmacokinetics *but this is molecule-specific*
- glycoform variations also lead to lot consistency problems & are challenging analytically

Studies on Restoring Sialylation

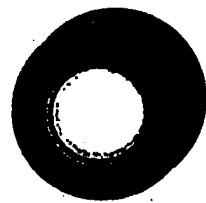
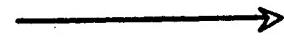
1. Transfect CHO cells with α 2,6-Sialyltransferase and monitor IFN- γ glycosylation and clearance.
2. Downstream re-sialylation of GPX by α 2,3-Sialyltransferase & CMP-NeuAc in vitro. Monitor GPX glycosylation and clearance.

Types of Transfection

CHO-320 (IFN)

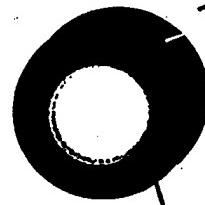


2,6-ST,
neo



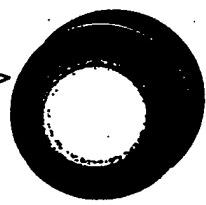
CHO-320 (IFN)

Dux-B11



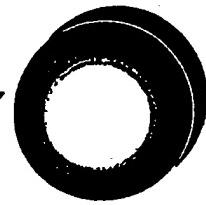
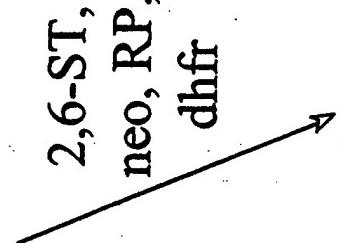
2,6-ST,
neo

RP, dhfr



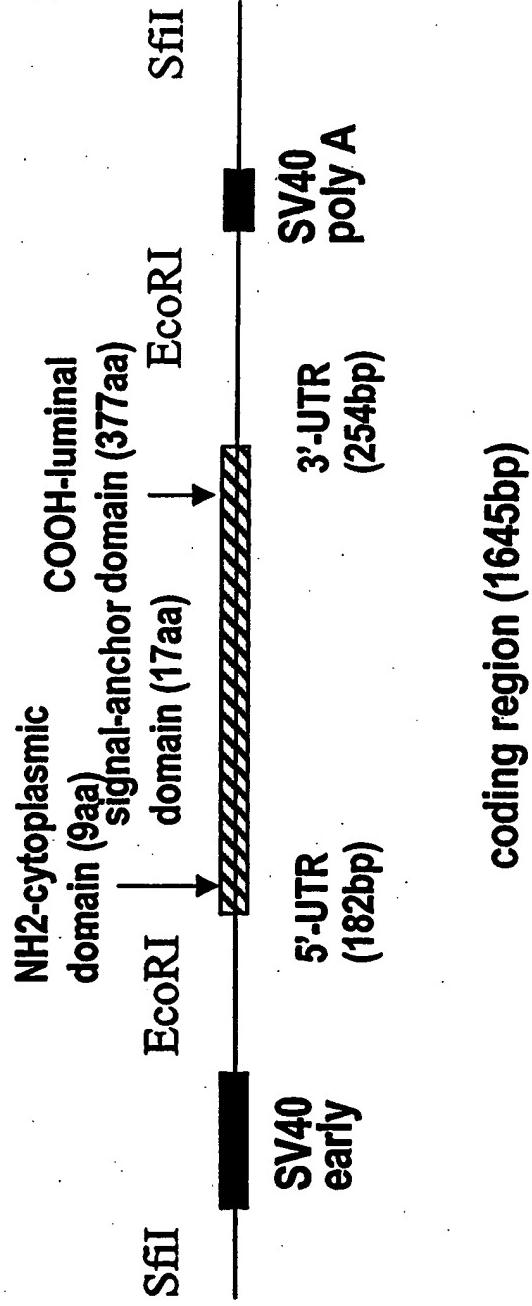
RP, dhfr

2,6-ST,
neo, RP,
dhfr

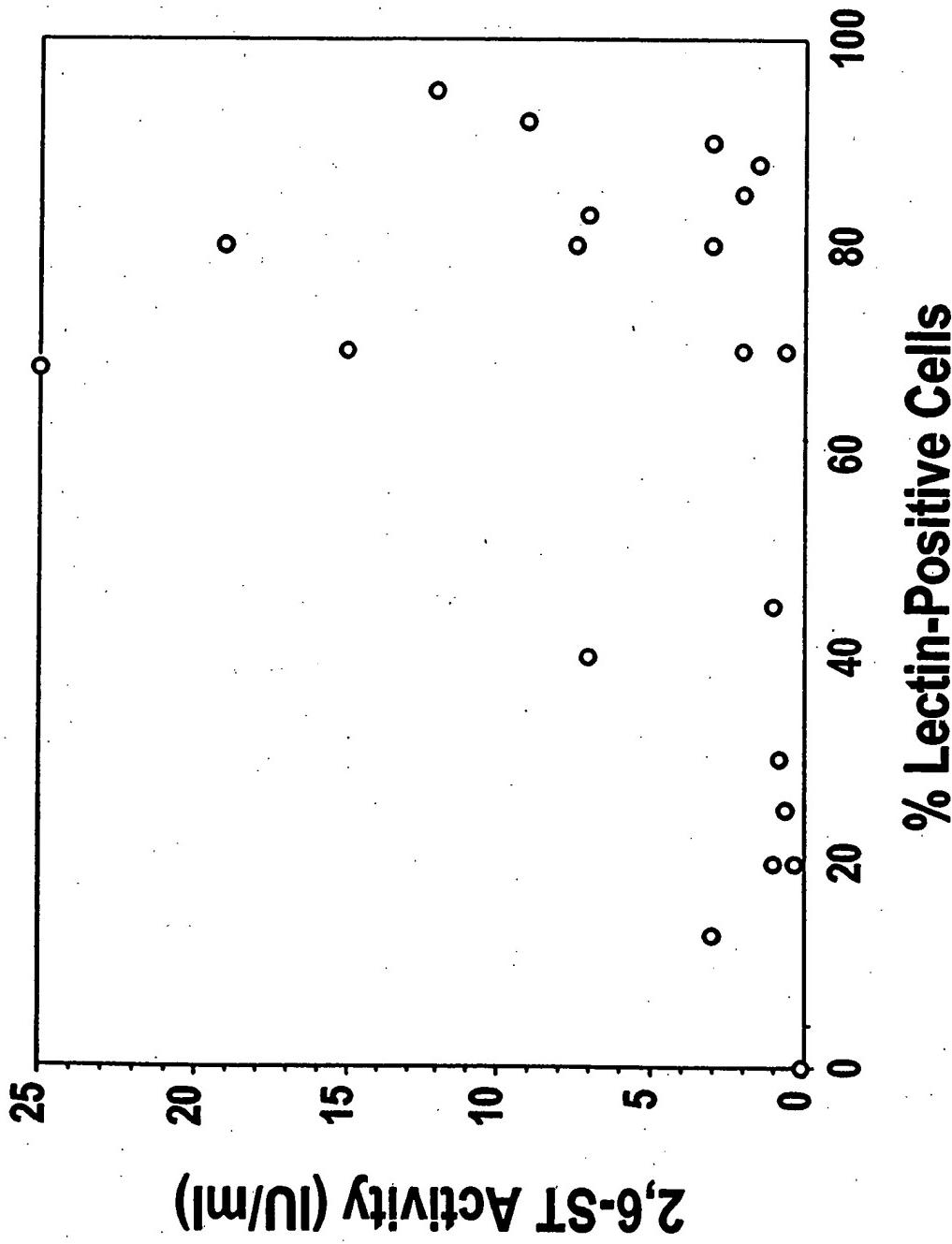


RP

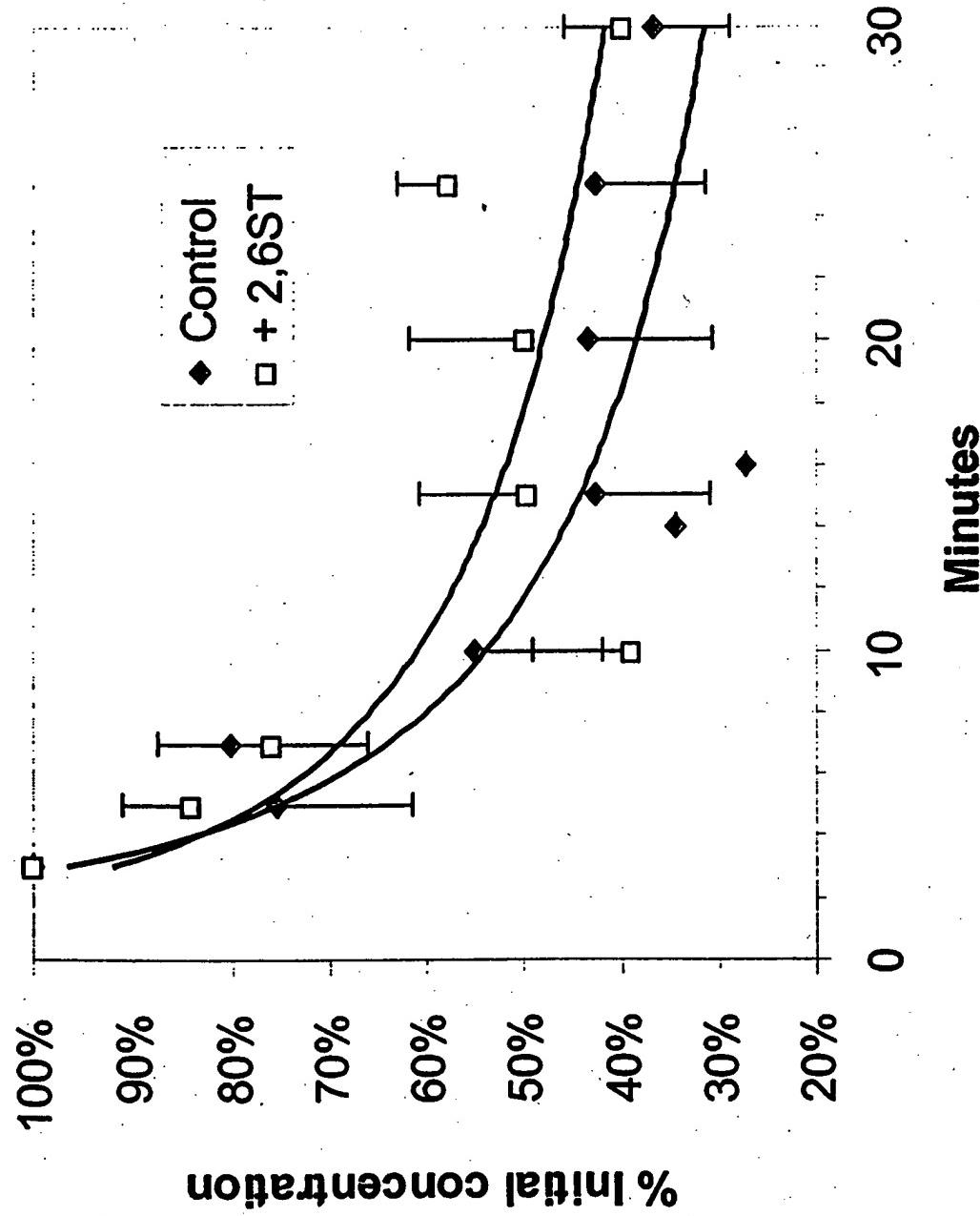
Rat α 2,6-Sialyltransferase Expression Cassette (3.5 kb)



FACS & Enzyme Assay Correlation



Clearance of IFN- γ Glycoforms in Rats



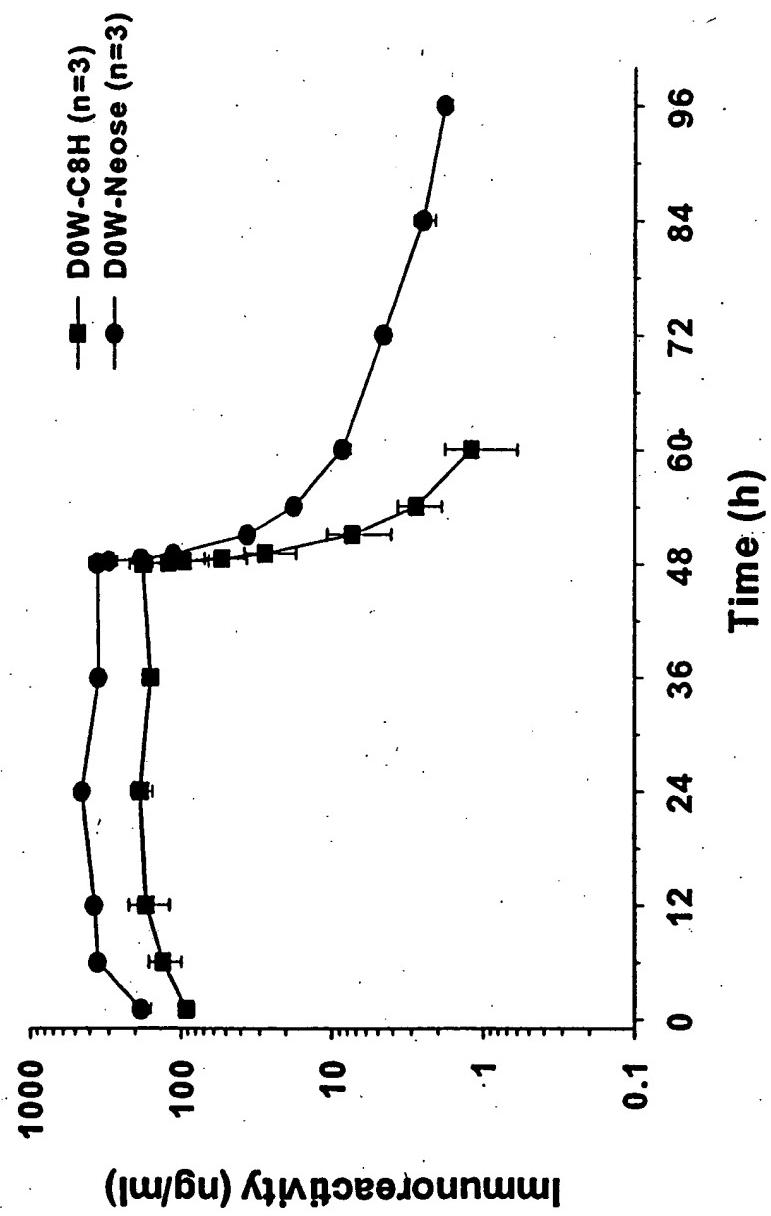
Re-Sialylation Study Design

- Glycoprotein X (GPX), a protein with 1 predominantly bi-antennary N-linked glycan and 1 O-linked glycan expressed in a mammalian cell line
- One lot of purified, under-sialylated GPX was sent to Neose for re-sialylation & compared to the original purified material
$$\text{2,3-Sialyltransferase}$$
$$\text{GPX-Gal + CMP-NeuAc} \longrightarrow \text{GPX-Gal-NeuAc}$$
- Both types of GPX were physically characterized by ESMIS & their pharmacokinetic profiles compared using bolus injections and continuous infusion into Cynomolgus monkeys

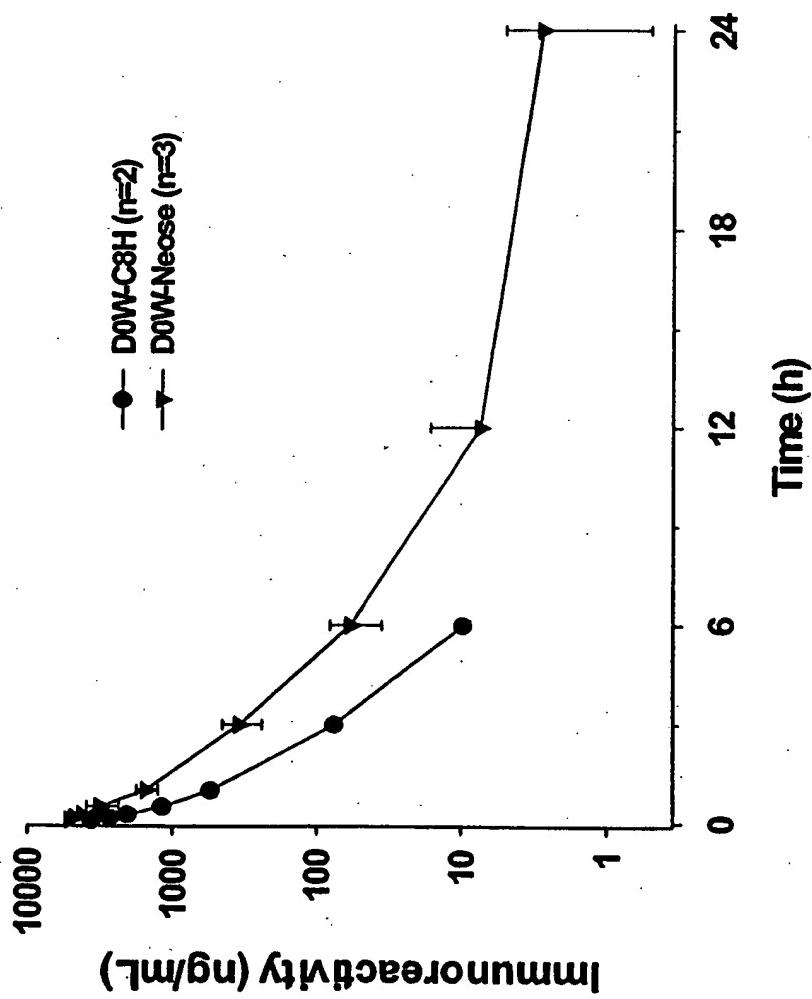
Carbohydrate Analysis by Electrospray MS

Property	Original material Dow-C8H	Modified material Dow-Neose	Theoretical Maximum
Sialic Acid Content (mol/mol protein)			
Total	0.84	3.12	3.79
O-Linked	0.61	0.82	1.41
N-Linked	0.23	2.30	2.38
% Sialylation			
Total	10	97	100
O-Linked	47	58	100
N-Linked	15	99	100
Under Galactosylation			
% Total	24.3	24.4	0
% O-linked	8.9	9.0	0

Continuous Infusion of 0.84 mg/Kg/d into Monkeys



Bolus Injection of 0.5 mg/Kg into Monkeys



Pharmacokinetic parameters after continuous intravenous infusion of 0.84 mg/kg/day for 48 hours

Parameter	Lot# D0W-C8H; (0.84 moles sialic acid/mole)	Lot# D0W-Neose; (3.1 moles sialic acid/mole)
C _{ss} (ng/mL) ^b	174.4 ± 32.3	380.0 ± 14.6
AUC _{0-96 hr} (ng·h/mL)	6889.5 ± 1354.7	16562.5 ± 1376.2
t _{1/2β} (h) ^c	3.93 ± 0.38	13.5 ± 0.52
t _{1/2α} (h) ^d	0.37 ± 0.09	0.70 ± 0.06
Clearance (L/h/kg)	0.25 ± 0.05	0.10 ± 0.01
V _{ss} (L/kg)	0.21 ± 0.01	0.21 ± 0.03

^a data are the mean± standard deviation (n=3/group)

^b C_{ss}, average of C_{max} values from 12, 24, 36 and 48 hours

^c elimination half-life
^d distribution half-life

Conclusions

- transfection of 2,6-ST into CHO cells changes sialic acid linkages but does not result in 100% sialylation. Differences in IFN clearance were observed.
- Re-sialylation was successful in restoring NeuAc on 99% of exposed Gal residues in N-linked glycans of GPX
- this process was ineffective on non-Gal residues (incomplete substrate) and O-linked glycans (different enzyme specificity)
- increasing N-linked sialylation of GPX leads to:
 - 2.5 fold slower plasma clearance & 2.2 fold increase in Css (steady-state plasma concentration) following constant infusion of 0.84 mg/Kg/d
 - 2.4 fold slower plasma clearance & 2.1 fold increased elimination half-life (t_{1/2b}) following bolus injection of 0.5 mg/Kg

Future Work

- evaluate purification & assay steps needed to ensure removal of Sialyltransferase & CMP-NeuAc from final GMP product
- assess scalability of enzyme steps & byproduct removal
- evaluate cost-effectiveness of Neose technology for individual proteins

Production of a complement inhibitor possessing sialyl Lewis X moieties by *in vitro* glycosylation technology

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Recombinant soluble human complement receptor type 1 (sCR1) is a highly glycosylated glycoprotein intended for use as a drug to treat ischemia-reperfusion injury and other complement-mediated diseases and injuries. sCR1-sLe^x produced in the FT-VI-expressing mutant CHO cell line LEC11 exists as a heterogeneous mixture of glycoforms, a fraction of which include structures with one or more antennae terminated by the sialyl Lewis X (sLe^x) [Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc] epitope. Such multivalent presentation of sLe^x was shown previously to effectively target sCR1 to activated endothelial cells expressing E-selectin. Here, we describe the use of the soluble, recombinant α 2-3 sialyltransferase ST3Gal-III and the α 1-3 fucosyltransferase FT-VI *in vitro* to introduce sLe^x moieties onto the N-glycan chains of sCR1 overexpressed in standard CHO cell lines. The product (sCR1-S/F) of these *in vitro* enzymatic glycan remodeling reactions performed at the 10-g scale has approximately 14 N-glycan chains per sCR1 molecule, comprised of biantennary (90%), triantennary (8.5%), and tetraantennary (1.5%) structures, nearly all of whose antennae terminate with sLe^x moieties. sCR1-S/F retained complement inhibitory activity and, in comparison with sCR1-sLe^x produced in the LEC11 cell line, contained twice the number of sLe^x moieties per mole glycoprotein, exhibited a twofold increase in area under the intravenous clearance curve in a rat pharmacokinetic model, and exhibited a 10-fold increase in affinity for E-selectin in an *in vitro* binding assay. These results demonstrate that *in vitro* glycosylation of the sCR1 drug product reduces heterogeneity of the glycan profile, improves pharmacokinetics, and enhances carbohydrate-mediated binding to E-selectin.

Key words: glycoengineering/glycoprotein remodeling/glycosylation/glycosyltransferase

Introduction

Soluble complement receptor type 1 (sCR1) is a recombinant glycoprotein that has been shown to inhibit the progression of the complement cascade in both the classical and alternative pathways by inhibiting the stable formation of C3 and C5 convertases and by serving as a cofactor in the proteolytic degradation of C3b and C4b by Factor I (Weisman *et al.*, 1990). The administration of sCR1 has been shown to be effective in a number of animal disease models of human complement-dependent ischemia-reperfusion injury for tissues, such as heart (Lazar *et al.*, 1999), liver (Lehmann *et al.*, 1998), hind limb (Kyriakides *et al.*, 2001a), lung (Naka *et al.*, 1997), and intestine (Williams *et al.*, 1999). Complement inhibition by sCR1 has been shown to reduce hyperacute rejection (Pruitt *et al.*, 1997) and enhance graft survival in many established transplant models (Kallio *et al.*, 2000; Pratt *et al.*, 1996; Stammberger *et al.*, 2000).

In some clinical situations, complement inhibition therapy could be more effective if it were targeted directly to sites of endothelial activation. At sites of inflammation, activated endothelial cells express E-selectin and P-selectin, surface adhesins with carbohydrate-binding domains that recognize the carbohydrate epitope, sLe^x (Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β 1-) (Lasky, 1995).

Previously we have described sCR1-sLe^x (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), a variant of the sCR1 glycoprotein conveniently produced in LEC11 cells transfected with the sCR1 gene. LEC11 is a mutant Chinese hamster ovary (CHO) cell line that expresses fucosyltransferase VI (FT-VI), a Golgi enzyme capable of adding fucose in α 1-3 linkage to GlcNAc in oligosaccharide chains that terminate with either Gal β 1-4GlcNAc β 1 ... or NeuAc α 2-3Gal β 1-4GlcNAc β 1 ... (Zhang *et al.*, 1999). Of the 25 potential N-glycosylation sites within the sCR1 polypeptide sequence, 13–15 are occupied, the majority with biantennary chains, creating the possibility for as many as 30 sLe^x moieties per molecule of sCR1-sLe^x. However, a previously reported analysis of the N-glycans of sCR1-sLe^x showed heterologous oligosaccharides with a variety of partially sialylated and fucosylated structures yielding less than the maximal number of sLe^x moieties (Picard *et al.*, 2000; Rittershaus *et al.*, 1999). Similar heterogeneity of glycans in CHO-expressed glycoproteins has been described previously and attributed to incomplete Golgi processing, post-secretion degradation due to glycohydrolases released into cell culture media, or both (Goochée *et al.*, 1991; Jenkins *et al.*, 1996).

In this article we describe a process to introduce sLe^x moieties onto the N-glycan chains of sCR1 produced in standard CHO cell lines using *in vitro* enzymatic synthesis.

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This method employs serial treatment of sCR1 with soluble recombinant rat ST3Gal-III and human FT-VI to give an sCR1-sLe^x product, designated sCR1-S/F (for differentiation from the LEC11 product) in which the antennae of N-glycans are nearly uniformly terminated with sLe^x epitopes. The benefits of *in vitro* glycan remodeling include improved pharmacokinetics, enhanced binding to E-selectin, and a means to improve product homogeneity. Enzymatic remodeling is demonstrated at the 10-g scale.

Results

In vitro remodeling of sCR1 glycans

sCR1 (250 mg) expressed in CHO cells was sialylated by treatment with ST3Gal-III plus CMP-sialic acid to give a product designated sCR1-S. After an aliquot was removed from the reaction mixture for analysis, the remaining sCR1-S was fucosylated in the same reaction vessel by the addition of FT-VI plus GDP-fucose to give a product designated sCR1-S/F. After purification by serial chromatography on ceramic hydroxyapatite and Q Sepharose, the reaction products had the same retention time and percent purity (98.5%) by reversed phase high-pressure liquid chromatography (RP-HPLC) as the starting material, sCR1 (data not shown). Chemical and functional properties of these molecules were compared with those of sCR1-sLe^x, a molecule previously produced in the FT-VI-expressing LEC11 CHO cell line and shown to contain some N-linked biantennary glycans terminated with the sLe^x tetrasaccharide (Picard *et al.*, 2000).

From the mannose content of sCR1, sCR1-S, sCR1-S/F, and sCR1-sLe^x (Table I) it may be inferred that these molecules contain ~13–15 N-glycan chains per mol protein (assuming 3 mol mannose per N-glycan chain). The fluorophore-assisted carbohydrate electrophoresis (FACE) oligosaccharide profile for sCR1 (Figure 1) shows three major bands consistent with a biantennary structure containing zero, one, or two sialic acid residues, as described previously (Picard *et al.*, 2000). The monosaccharide composition of sCR1 (Table I) suggests that ~57% of total

galactosyl residues are substituted with sialic acid (19 mol sialic acid/ 33.2 mol galactose). By comparison, the FACE oligosaccharide profile for sCR1-S (Figure 1) shows one major band that migrates at a position consistent with a biantennary structure containing two sialic acid residues, and monosaccharide analysis reveals the galactose/sialic acid ratio to be 1:1 (Table I).

FACE analysis of glycans from sCR1-S/F, prepared by enzymatic fucosylation of sCR1-S, suggests that N-glycans are predominantly biantennary and that fucosylation at both antennae is nearly complete (Figure 1). The dominant oligosaccharide band derived from sCR1-S/F was cut out and extracted from the gel. Sequential removal of monosaccharide residues from the extracted glycoprotein using specific glycosidases gave products with mobilities consistent with α 1-6 core-fucosylated, biantennary N-glycans (Figure 2). Monosaccharide analysis of sCR1-S/F shows the presence of 39.3 moles fucose per mol sCR1-S/F, a figure in agreement with the prediction from theory that 39–45 fucose residues per mol protein would be present if all N-glycans were core fucosylated and enzymatic fucosylation of antennary GlcNAc residues were complete.

The FACE oligosaccharide profile for sCR1-sLe^x, a glycoprotein produced in LEC11 CHO cells, shows at least seven bands (Figure 1) with some common to sCR1 and others shown previously (Picard *et al.*, 2000) to represent core fucosylated structures with α 1-3 fucosylation at one or more antennae. Heterogeneity in the degree of fucosylation of the N-glycan chains from sCR1-sLe^x also can be appreciated from the results of monosaccharide analysis (Table I). For example, it may be calculated (assuming 3 mannose residues per chain) that sCR1-sLe^x contains an average of 2.5 fucosyl residues per glycan chain. By contrast, the fucose content per glycan chain increases from 0.95 for

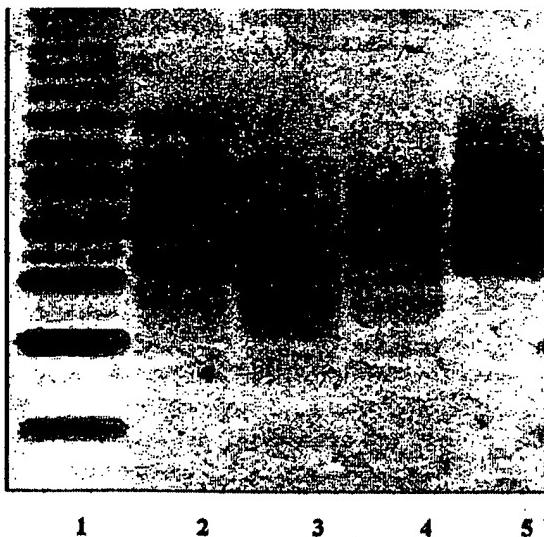


Fig. 1. FACE profiling of oligosaccharides from sCR1-sLe^x and sCR1 before and after enzymatic remodeling: (1) Glyko oligosaccharide standard ladder, (2) sCR1, (3) sCR1-S, (4) sCR1-S/F, (5) sCR1-sLe^x. The oligosaccharide profile of sCR1 (lane 2) contains predominantly bands representing biantennary structures with two sialic acids (bottom band), one sialic acid (middle band), and no sialic acids (top band).

Table I. Monosaccharide Content (mol/mol glycoprotein) by HPLC analysis

	sCR1	sCR1-S	sCR1-S/F	sCR1-sLe ^x
Glucosamine	62	48	48	62
Galactose	33	28	27	38
Mannose	44	39	35	40
Fucose	16	12	39	33
Sialic acid	19	30	28	27
Sialic acid/galactose	0.57	1.09	1.06	0.70
Glycosylation sites/sCR1	15	13	12	13
Estimated sLe ^x /sCR1-sLe ^x * n.a.	n.a.	28	14	

*Estimated sLe^x/sCR1-sLe^x = (Fuc/sCR1-sLe^x – sites/sCR1-sLe^x) × Sial/Gal ratio.

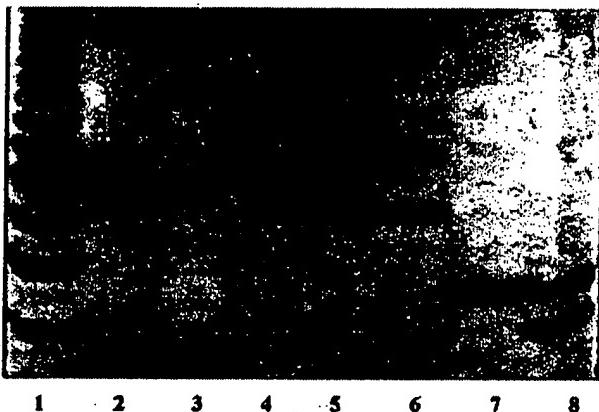


Fig. 2. FACE analysis of oligosaccharides from sCR1-S/F after serial treatment with glycosidases. The dominant oligosaccharide band derived from sCR1-S/F (lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was digested sequentially to remove each monosaccharide residue starting at the terminal sialic acid residue and ending at the trimannosyl core: (1) Glyko oligosaccharide standard ladder; (2) total N-linked oligosaccharides of sCR1-S/F; (3) purified dominant band (band 1) from lane 2; (4) band 1 treated with NANaseIII (cleaves α 2-3, 4, 6, 8, and 9 linked sialic acid); (5) band 1 treated with NANaseIII and FucaseIII (cleaves α 1-3 and 4 fucose); (6) band 1 treated with NANaseIII, FucaseIII, and GalaseIII (cleaves terminal galactose); (7) band 1 treated with NANaseIII and FucaseIII, GalaseIII, and hexosaminidase; (8) standard trimannosyl core N-glycans with (upper band) and without α 1-6 fucose.

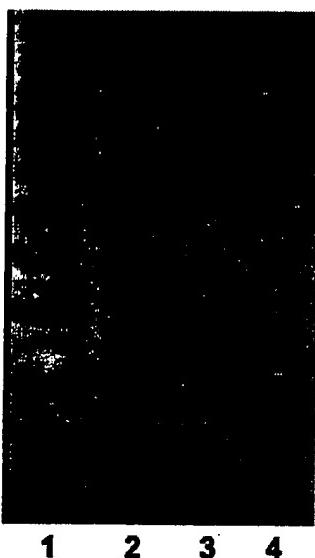


Fig. 3. FACE analysis of oligosaccharides from sCR1-S/F treated with sialidases. The dominant oligosaccharide band (band 1) derived from sCR1-S/F (see Figure 2, lane 2) was cut out and extracted from the gel. The resulting oligosaccharide preparation was subjected to enzymatic digestion to remove terminal sialic acid: (1) Glyko oligosaccharide standard ladder; (2) band 1 from sCR1-S/F; (3) band 1 treated with NANaseI (cleaves α 2-3 linked sialic acid); (4) band 1 treated with NANaseIII (cleaves α -3, 4, 6, 8, and 9 linked sialic acid).

sCR1-S to 3.3 for sCR1-S/F, a result that correlates well with the single band visualized by FACE analysis of sCR1-S/F (Figure 1).

Oligosaccharide sequencing using FACE

The linkage of terminal sialic acids on sCR1-S/F was assessed by digestion with specific neuraminidases (Figure 3). Complete removal of sialic acid by treatment of band 1 from sCR1-S/F with NANase I indicates that sialic acid residues are α 2-3 linked, as expected.

Optimization of sialylation reaction for scale-up

To establish conditions for scaleup of sialylation, sCR1 (5 mg/ml) was incubated with varying amounts of ST3Gal-III (10, 25, 75, 100, 200, 300 and 400 U/ml) and 5 mM CMP-sialic acid plus a trace amount of radiolabeled CMP-sialic acid for 24 h at 32°C. At an ST3Gal-III concentration of 150 mU/ml, incorporation of radiolabeled sialic acid reached 91% of maximum after 24 h and 100% at 48 h. The lowest concentration of enzyme required to give nearly maximum incorporation (~40 mol sialic acid/mol protein) under these conditions was 25 mU/ml ST3Gal-III (Figure 4). It should be noted that the contribution of triantennary and tetraantennary species may be responsible for the observation that more than 30 moles of sialic acid was added per mole of sCR1. Increasing the CMP-sialic acid concentration from 5 mM to 10 mM did not affect the level of sialylation of sCR1 at any of the ST3Gal-III concentrations tested (data not shown). HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of glycans

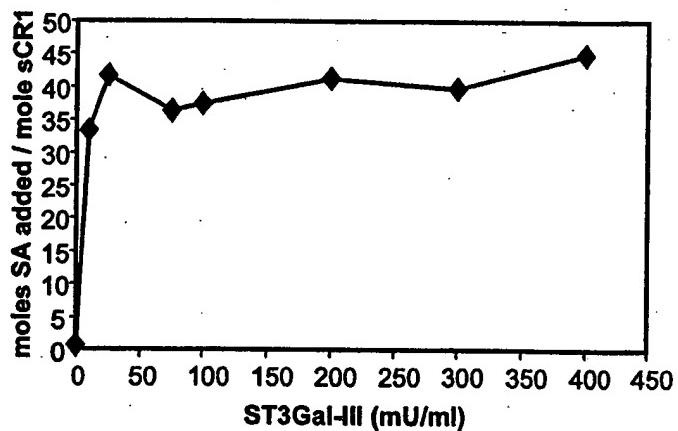


Fig. 4. Incorporation of sialic acid into sCR1 at increasing concentrations of ST3Gal-III in a 24-h reaction. The moles of sialic acid added are estimated from incorporation of radiolabeled CMP-sialic acid. Incorporated radiolabel is separated from free by gel filtration on a TSKG2000_{SWXL} column.

released from sCR1-S revealed that at all concentrations of enzyme tested, the product contained predominantly disialylated, biantennary, core fucosylated N-glycans (data not shown). A concentration of 200 mU ST3Gal-III/ml was chosen for scale-up to ensure completeness of reaction.

Optimization of fucosylation

To establish conditions for scale-up of fucosylation, sCR1-S (5 mg/ml) was incubated with varying amounts of

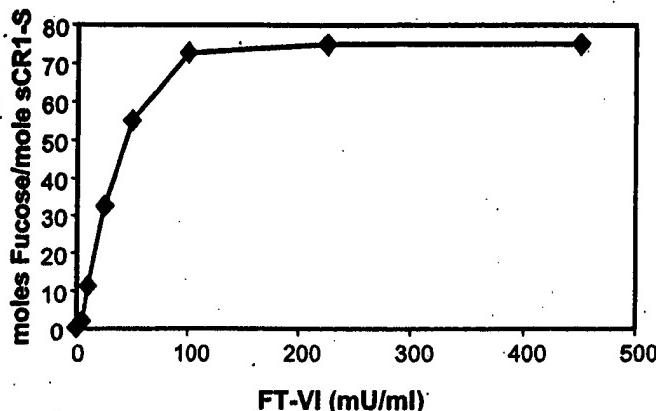


Fig. 5. Incorporation of fucose into sCR1-S at increasing concentrations of FT-VI in a 24-h reaction. The moles of fucose added are estimated from incorporation of radiolabeled GDP-fucose. Incorporated radiolabel is separated from free by gel filtration on a TSKG2000_{SWXL} column.

FT-VI (10, 20, 40, 60, 100, 220, 440 mU/ml) and 5 mM GDP-fucose plus a trace of radiolabeled GDP-fucose for 24 h at 32°C. The lowest concentration of enzyme required to give nearly maximum incorporation of fucose under these conditions was 100 mU/ml FT-VI (Figure 5). Increasing the GDP-fucose concentration from 5 mM to 10 mM did not increase fucose incorporation at several different FT-VI concentrations tested (data not shown).

For products of reactions run at all concentrations of FT-VI \geq 100 mU/ml, the glycan structures identified by HPLC and MALDI-TOF MS were almost the same and essentially indistinguishable from the structures described next for sCR1-S/F produced at the 10-g scale.

Remodeling at 10-g scale

Purified sCR1 (10 g in a volume of 2 L) was incubated first with ST3Gal-III plus CMP-sialic acid at 32°C for 36 h and then, following addition of FT-VI plus GDP-fucose, incubated at 32°C for another 36-h period..

FACE analyses of glycans from sCR1, sCR1-S, and sCR1-S/F for reactions performed at the 10-g scale (data not shown) were essentially indistinguishable from FACE results obtained at the 250-mg scale (Figure 1), suggesting that occupancy of potential acceptor sites for ST3Gal-III and FT-VI on sCR1 at the 10-g scale was nearly complete.

HPLC profiles for 2-AA-derivatized glycans of sCR1, sCR1-S, and sCR1-S/F are shown in Figure 6 and the percentages of glycan species estimated from integrated peak areas are summarized in Table II. After *in vitro* sialylation with ST3Gal-III, neutral glycans, comprising 50% of carbohydrate chains in sCR1, are reduced to 2% of chains in sCR1-S, and monosialo-glycans likewise decrease to from 35% in sCR1 to 17.5% in sCR1-S (Figure 6 and Table II). Overall, about 90% of N-glycans are biantennary and these chains contain an average of 1.8 sialic acid moieties per glycan. Among the minority of biantennary glycans on sCR1-S that are monosialylated, some lack galactose on one antenna, whereas others contain two galactosyl residues, only one of which is sialylated. The remaining 10% of

Table II. HPLC data summary of large scale remodeling

Glycan species	Native protein (sCR1) (%)	Sialylated protein (sCR1-S) (%)	Sialylated and fucosylated protein (sCR1-S/F) (%)
Neutral	50.5	2.0	4.0
1 charge	35.0	17.5	25.5
2 charges	13.0	70.5	68.5
3 charges	1.5	8.5	1.5
4 charges	ND*	1.5	0.5

*Not detected.

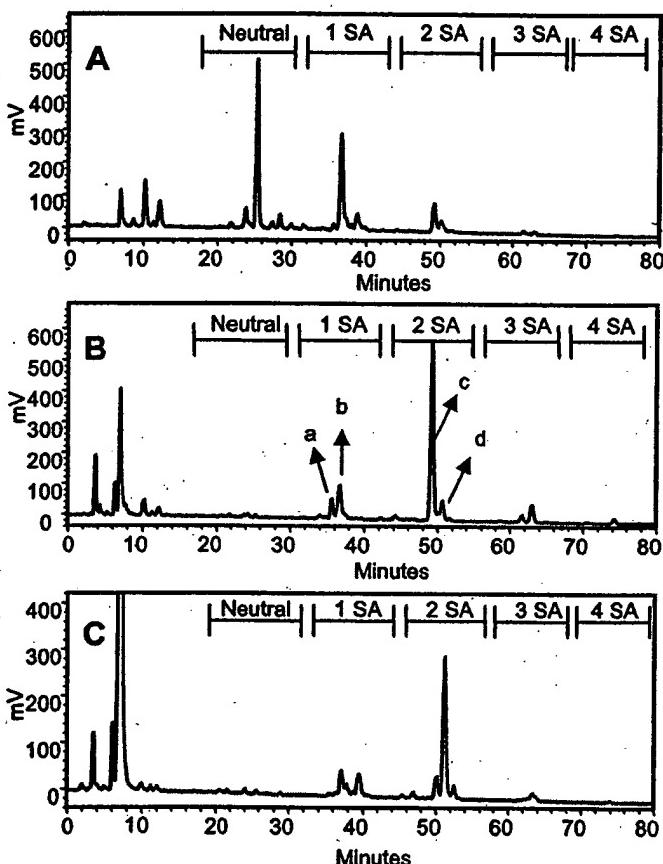


Fig. 6. RP-HPLC analysis of 2-AA-oligosaccharides before and after enzymatic remodeling at the 10-g scale: (A) sCR1; (B) sCR1-S; (C) sCR1-S/F. MALDI-TOF MS analysis (data not shown) of 2-AA-oligosaccharides from sCR1-S (B) indicated that: peak a contains monosialylated biantennary glycans that lack terminal galactose on one antenna; peak b, constituting 12% of biantennary glycans, contains biantennary glycans with two galactose residues, but only one sialic acid; peaks c and d contain disialylated, biantennary glycans, with and without core fucose, respectively.

glycans are fully sialylated triantennary (8.5%) or tetra-antennary (1.5%) structures.

After fucosylation of sCR1-S to create sCR1-S/F, HPLC and MALDI-TOF MS analyses (Table III and Figure 7)

Table III. sCR1-S/F glycans

Neutral glycans		Neutral glycans		Monosialo glycans		Disialo glycans	
structure	%	structure	%	structure	%	structure	%
	0.64		0.16		0.70		17.26
	0.16		0.13		3.47		51.24
	0.44		0.38		1.06		
	0.43		1.51		1.15		
	0.18		0.82		7.07		
	0.95				2.93		
	0.28				6.12		
	0.35						
	0.58						

Trisialo glycans	
structure	%
	0.75
	0.75

Blue squares represent N-acetylglucosamine, yellow circles represent mannose, green triangles represent fucose, red diamonds represent galactose, and asterisks represent sialic acid.

showed that more than 95% of the glycans were fucosylated by FT-VI. About 62% of the total N-glycans gained two fucose residues, and ~30% gained a single fucose residue. Failure to accept two fucosyl residues was in part due to missing galactosyl residues on one or more antennae. From these results it can be estimated that the sCR1-S/F molecules created by consecutive *in vitro* sialylation and fucosylation reactions contain, on average, 28 sLe^x epitopes per protein molecule, whereas sCR1-sLe^x, glycosylated and secreted by the FT-VI-expressing LEC11 CHO cell, contains ~14 sLe^x epitopes per protein molecule (Table I).

To check the stability of sCR1 under conditions of incubation with glycosyltransferases, a small amount of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after each remodeling reaction. There was no evidence of degradation of the

polypeptide after incubation with either ST3Gal-III or FT-VI (data not shown).

Pharmacokinetics

When sCR1-S prepared at the 250-mg scale was injected intravenously into rats, the observed area under the curve (AUC_{last}) was twofold greater than the AUC_{last} for sCR1 ($p < 0.004$), indicating a significantly greater exposure of the more completely sialylated form of the complement inhibitor to intravascular cells following dosing (Figure 8).

In vitro antihemolytic activity

The IH₅₀ values for sCR1, sCR1-S, sCR1-sLe^x, and sCR1-S/F as inhibitors of human complement-mediated lysis of sheep red blood cells were found to be similar (Figure 9 and

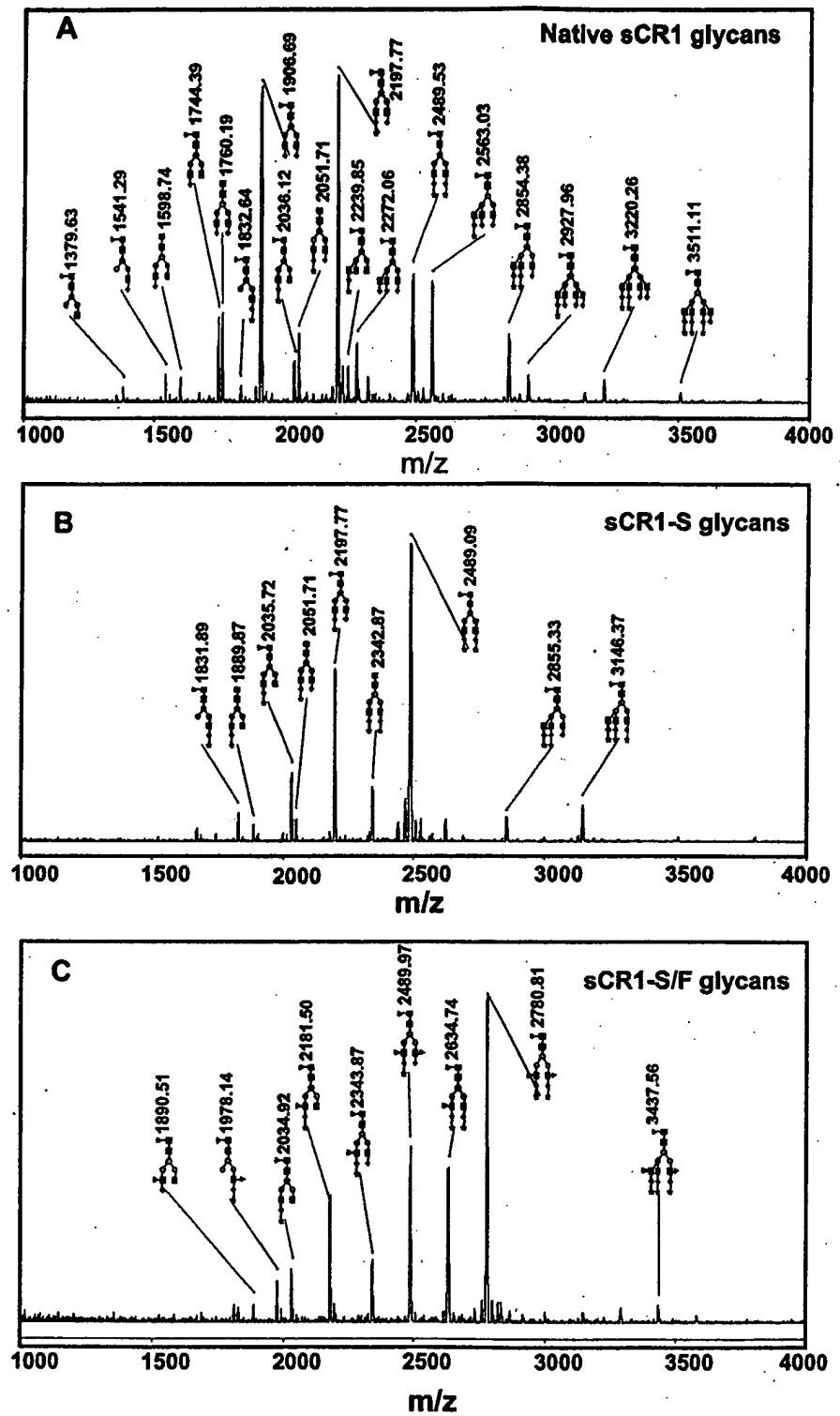


Fig. 7. MALDI-TOF analysis of total glycans from (A) sCR1, (B) sCR1-S, and (C) sCR1-S/F remodeled at the 10-g scale. The blue square is GlcNAc, the yellow filled circle is mannose, the green filled triangle is fucose, the red filled diamond is galactose, and the asterisk is sialic acid.

Table IV), indicating that *in vitro* glycosylation of sCR1 to yield sCR1-S or sCR1-S/F does not significantly impact the complement inhibitory properties of the molecule in the classical pathway.

In vitro binding to E-selectin

Figure 10 shows that sCR1-sLe^x and sCR1-S/F bind E-selectin in a concentration-dependent manner. The IC₅₀

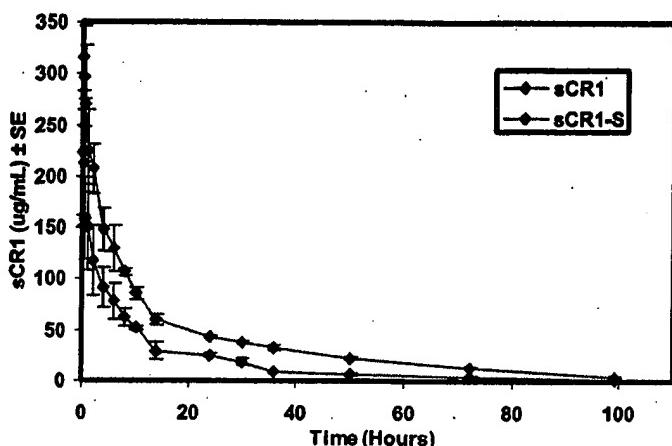


Fig. 8. The concentration of sCR1 and sCR1-S in plasma at various time points following bolus IV injection in rats.

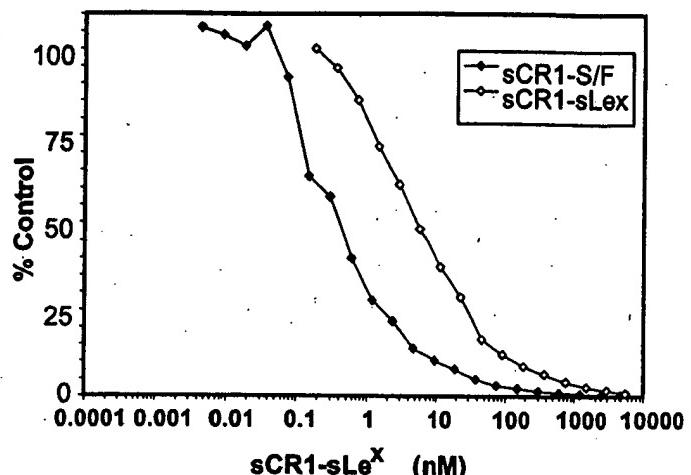


Fig. 10. Inhibition of PAA-sLe^X binding to E-selectin coated microtiter plates in the presence of varying concentrations of sCR1-sLe^X or sCR1-S/F.

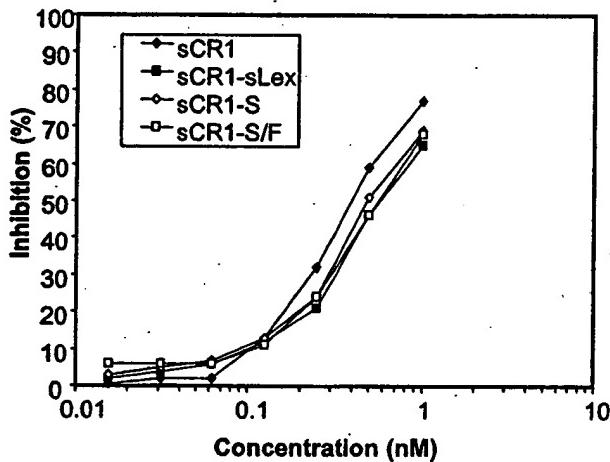


Fig. 9. Inhibition of red cell lysis via the classical pathway as a function of the concentration of sCR1, sCR1-S, sCR1-sLe^X, and sCR1-S/F.

Table IV. Antihemolytic activity of modified sCR1 and sCR1-sLe^X

	IH ₅₀ (nM)
sCR1	0.41
sCR1-S	0.48 nM
sCR1-sLe ^X	0.59
sCR1-S/F	0.59 nM

for sCR1-sLe^X from this plot is ~5 nM, and for sCR1-S/F ~0.4 nM. The observed 10-fold increase in inhibitory potency presumably is due to enhanced avidity, attributable to the increased density of sLe^X moieties on sCR1-S/F (28/mol) as compared with sCR1-sLe^X (14 per mol) (see Table I). The specificity of this binding was demonstrated by its calcium requirement and by the observation that sCR1

(which does not contain any sLe^X structures) does not inhibit E-selectin binding at concentrations as high as 10 μM (data not shown).

Discussion

sCR1, made by standard CHO production methods, possesses predominantly biantennary oligosaccharides that are incompletely sialylated. We previously described an alternately glycosylated form of sCR1 called TP20 or sCR1-sLe^X (Picard *et al.*, 2000; Rittershaus *et al.*, 1999), secreted by the FT-VI-expressing LEC11 CHO cell line and bearing sLe^X moieties on a fraction of its N-linked oligosaccharides. In this article we describe *in vitro* enzymatic remodeling of sCR1 by the stepwise application of two soluble recombinant glycosyltransferases in "one pot": The first step adds sialic acid to make sCR1-S, and the second adds fucose to make sCR1-S/F. The product of these glycan remodeling reactions contains an average of 28 sLe^X moieties per mol, as compared with 14 per mol found in CHO cell-produced sCR1-sLe^X.

That the sCR1 protein remains intact under conditions of glycan remodeling was demonstrated by RP-HPLC and SDS-PAGE analyses showing single polypeptides with expected molecular weights for sCR1-S and sCR1-S/F. Evidence for (1) conformational stability under conditions of the *in vitro* glycosylation reactions, and (2) preserved function despite variations in glycan structure, is provided by the observed near equivalence in bioactivity of sCR1, sCR1-S, sCR1-S/F, and CHO-produced sCR1-sLe^X in a standard complement inhibition assay.

The oligosaccharide structures associated with sCR1-S and sCR1-S/F were assessed by a number of methods. FACE profiling demonstrated a more fully sialylated set of glycoforms for sCR1-S as compared with sCR1 and nearly homogeneous, fully sialylated and fucosylated biantennary N-glycans for sCR1-S/F. Sequencing experiments using FACE provided supporting evidence that sialic acid

was linked α 2-3 to galactose and that the predominant, single oligosaccharide band derived from sCR1-S/F was BiNA₂F₂. The analyses we performed do not establish linkages between the terminal and penultimate sugars that define sLe^x (NeuAco2-3Galβ1-4[Fucα1-3]GlcNAcβ1-) versus sLe^a (NeuAco2-3Galβ1-3[Fucα1-4]GlcNAcβ1-). However, two factors make it likely that the glycans of SCR1-S/F do, in fact, terminate in sLe^x. First, it is known that in CHO cells, N-linked glycans are most commonly formed by β4GALT-1, and hence have the type-2 structure, Galβ1-4GlcNAcβ1- (Lee et al., 2001). Second, the acceptor specificity of FT-VI is known to be restricted to type 2 chains (Costache et al., 1997; Weston et al., 1992).

During optimization of the sialylation reaction, we noted that incubation of sCR1 with either a low concentration of ST3Gal-III (10 mU/ml) for 24 h or a higher concentration (75 mU/ml) for 1 h produced a nearly maximally sialylated product. Even after incubation at the highest concentration of sialyltransferase tested (600 mU/ml for 24 h), a small fraction of monosialylated biantennary species persisted, perhaps due to steric hindrance at particular sites. Improved pharmacokinetics observed for the fully sialylated sCR1-S molecule as compared with sCR1 is probably a consequence of the added sialic acid blocking the interaction of terminal galactosyl residues with hepatic asialoglycoprotein receptors (Stockert, 1995).

We observed that FT-VI at 25 mU/ml fucosylates most sialylated biantennary glycans within 24 h. No significant differences were observed in catalytic activities of FT-VI expressed in the NSO cell line versus *Aspergillus niger* expression systems. The sCR1 polypeptide was shown to be stable following prolonged incubation with enzyme from either source.

In vitro glycosylation of sCR1 at the 10-g scale was carried out at enzyme concentrations selected to ensure nearly complete reaction at each stage. Success with the single experiment reported is consistent with the ability to predict useful scaled-up reaction conditions over a range of at least 40-fold based on mass of starting substrate. Both the ST3Gal-III and FT-VI enzymes used to glycosylate 10 g sCR1 were produced in *A. niger*, an expression system widely used for the manufacture of industrial enzymes in ton quantities. Although further scale-up would require refinement of incubation conditions, it can be estimated from present results that glycosylation of 1 kg of sCR1 might require 40,000 U ST3Gal-III and 20,000 U FT-VI, amounts that seem plausible to produce at reasonable cost in an industrial setting. To our knowledge, this is the largest scale reported enzymatic glycosylation of a glycoprotein to date by several orders of magnitude (Fischer and Dorner, 1998; Nemansky et al., 1995; Paulson et al., 1977; Raju et al., 2001; Thotakura et al., 1994).

The optimized conditions chosen for scale-up were very similar to the conditions used to generate material used for *in vivo* and *in vitro* studies. Compared with sCR1-sLe^x, sCR1-S/F was shown to have twice the number of sLe^x moieties and about a 10-fold higher apparent affinity for binding to E-selectin. This higher affinity presumably results from increased cooperativity in a multivalent binding reaction wherein sLe^x moieties distributed widely over SCR1-S/F engage multiple immobilized E-selectin molecules. In certain

clinical situations, the anticomplement inhibitory and anti-inflammatory activity of sCR1-S/F could be effectively targeted via a similar mechanism to sites of inflammation where endothelial cells have been activated and have up-regulated expression of adhesion molecules including P- and E-selectin. sCR1-sLe^x has been shown to be superior to sCR1 in a complement- and selectin-dependent lung injury model (Mulligan et al., 1999), a murine model of ischemic stroke (Huang et al., 1999), moderating skeletal muscle reperfusion injury (Kyriakides et al., 2001a), moderation of acid aspiration injury (Kyriakides et al., 2001b), reducing ischemia/reperfusion injury in rat lung grafts (Schmid et al., 2001), and a myocardial ischemia and reperfusion model in the rat. sCR1-sLe^x significantly reduced myocardial infarct size and was significantly more effective than sCR1 in reducing neutrophil infiltration into the infarction (Zacharowski et al., 1999). It will be interesting to investigate whether sCR1-S/F is even more effective than sCR1-sLe^x in similar animal models.

Materials and methods

Complement proteins, antibodies, enzymes, and other reagents

Purified sCR1 and sCR1-sLe^x were prepared as previously described (Rittershaus et al., 1999). Nucleotide sugars (CMP-sialic acid and GDP-fucose) were manufactured at Neose (Horsham, PA). CMP-sialic acid was prepared from CTP and sialic acid with recombinant CMP NeuAc synthetase (Shames et al., 1991). GDP-fucose was either made from GDP-mannose using GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase/reductase, or purchased from Yamasa (Chiba, Japan). A gene encoding for a truncated, soluble form of ST3Gal-III (rat) was expressed in *A. niger* var. *awanori* dgr246 P2 using a variant of the expression vector pSL 1180 (Ward and Power, 2003). A 30–60% ammonium sulfate pellet was dissolved in 100 mM NaCl, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with 1 M NaCl, 20 mM MES, pH 6. rFT-VI (human) was expressed either in NSO cells or in *A. niger* as described as a soluble protein lacking the transmembrane domain. For the *A. niger* expressed protein, a 30–60% ammonium sulfate pellet was dissolved in 20 mM MES, pH 6, loaded on SP Sepharose (Amersham Biosciences, Piscataway, NJ), and eluted with a linear gradient from 0 to 1 M NaCl in 20 mM MES, pH 6. Soluble recombinant E-selectin was purchased from R&D Systems (Minneapolis, MN). Streptavidin–horseradish peroxidase conjugate (SA-HRP) was from Pierce (Rockford, IL), and biotinylated polyacrylamide polymer (PAA-sLe^x) was from GlycoTech (Rockville, MD). Anti-sCR1 monoclonal antibodies 6B1.H12 and 4D6.1 were prepared as previously described (Nickells et al., 1998). Standards and glycosidases used in FACE analyses were from Glyko (Novato, California).

Preparation of sCR1-S

Lyophilized sCR1 (250 mg) was reconstituted and buffer exchanged into 50 mM Tris, 0.15 M NaCl, 0.05% NaN₃,

pH 7.2, using gel filtration columns (PD-10, Amersham Biosciences), and the concentration of sCR1 was adjusted to 5 mg/ml with the same buffer. Following addition of ST3Gal-III (150 mU/ml) and CMP-sialic acid (7 mM) the mixture was incubated at 32°C. A separate aliquot of the reaction mixture to which a trace amount of CMP-[¹⁴C]sialic acid was added was incubated in parallel. From this, aliquot samples were withdrawn at various times and fractionated by isocratic HPLC/size-exclusion chromatography at 0.5 ml/min in 45% MeOH, 0.1% trifluoroacetic acid (7.8 mm × 30 cm TSKG2000swXL column, particle size 5 μm, TosoHaas). Incorporation of sialic acid into glycoprotein was calculated from the fraction of counts in the first eluted peak and the known concentration of sugar nucleotide.

Preparation of sCR1-S/F

After the sialylation reaction had proceeded for 48 h, GDP-fucose was added to a final concentration of 7 mM, MnCl₂ to 5 mM, and rFT-VI to 0.1 U/ml. A trace amount of GDP-[¹⁴C]fucose was added to a separate aliquot, and both reaction mixtures were incubated at 32°C. Chromatography of the radiolabeled mixture as described showed the transfer of ~44 moles/mole sCR1-S after 48 h and 47 moles after 48 h. The product was provisionally designated sCR1-S/F.

Removal of nucleotide sugars and residual glycosyltransferases using ceramic hydroxyapatite and Q Sepharose chromatography

Glycosyltransferases and nucleotide sugars were removed from remodeled sCR1-S and sCR1-S/F by chromatography on ceramic hydroxyapatite (type I; BioRad, Hercules, CA) followed by Q Sepharose (Amersham Biosciences). Purity was assessed by RP-HPLC on a Poros R1/10 column (4.6 mmD/100 mmL, Applied Biosystems, Framingham, MA).

Optimization of sialylation and fucosylation reactions prior to scale-up

sCR1 was thawed slowly at 4°C and buffer exchanged into 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, using a PD10 column. *In vitro* sialylation of sCR1 (5 mg/ml) was evaluated using varying amounts of ST3Gal-III, 5 mM CMP-sialic acid, in the presence of 0.02% sodium azide at 32°C for 24 h. A trace amount of CMP-[¹⁴C]sialic acid was added to an aliquot to monitor incorporation of radioactive sialic acid as described.

To the product (sCR1-S) of the reaction performed at a sialyltransferase concentration of 100 mU/ml (still containing the sialylation reagents) was added MnCl₂ and GDP-fucose, each to a final concentration of 5 mM, varying amounts of FT-VI, and a trace amount of GDP-[³H]fucose. The resulting reaction mixture was incubated at 32°C for 24 h. Incorporation of radioactive fucose into the product (sCR1-S/F) was monitored as described for sialic acid.

sCR1 remodeling at 10-g scale

Purified sCR1 (10 g) was dialyzed exhaustively at 4°C against 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, adjusted to a concentration of 5 mg/ml with the same buffer, and incubated with ST3Gal-III (200 mU/ml) and CMP-sialic

acid (5 mM) for 36 h at 32°C in a final volume of 2 L. After 36 h, an aliquot containing the sialylated product (sCR1-S) was withdrawn for analysis and the following reagents (final concentrations) were added: rFT-VI (100 mU/ml), GDP-fucose (5 mM), MnCl₂, (5 mM). After further incubation at 32°C for 36 h, a precipitate (manganese phosphate) was removed by centrifugation at 3000 × g for 5 min, and the sialylated and fucosylated product (sCR1-S/F) was stored at -70°C.

Monosaccharide analysis by HPLC

The neutral and amino sugar composition of glycoproteins was determined after trifluoroacetic acid hydrolysis and reductive amination with anthranilic acid by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1994). Sialic acid content was determined after sodium bisulfate hydrolysis and reaction with o-phenylenediamine by C18 reverse-phase HPLC with fluorescence detection (Anumula, 1995).

Carbohydrate analysis by FACE

Carbohydrate sequencing and electrophoresis by FACE (Glyko and ProZyme, San Leandro, CA) was performed as previously described elsewhere (Picard *et al.*, 2000).

Carbohydrate analysis by 2-AA HPLC and MALDI-TOF MS

Glycans were released by PNGaseF and labeled with 2-AA according to the method described by Anumula and Dhume (1998) except that the labeled glycans were purified on cellulose cartridges (Glyko) according to the manufacturer's instructions. 2-AA-labeled N-glycans were analyzed using a Shodex Asahipak NH₂-P-50 4D amino column (4.6 mm × 150 mm). The two solvents used for the separation were (A) 2% acetic acid and 1% tetrahydrofuran in acetonitrile and (B) 5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water. The column was eluted isocratically with 70% A for 2.5 min, followed by a linear gradient from 70% to 5% A over a period of 97.5 min, and a final isocratic elution with 5% A for 15 min. Eluted peaks were detected using fluorescence detection with an excitation wavelength of 230 nm and an emission wavelength of 420 nm.

For MALDI-TOF analysis, a small aliquot of the 2-AA-labeled N-glycans was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 μm pore, 47 mm diameter) floating on water. The dialyzed aliquot was dried in a vacuum centrifuge, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water:acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Glycan structures were assigned based on the observed mass-to-charge ratio and literature precedence. No attempt was made to fully characterize isobaric structures.

SDS-PAGE

sCR1 samples before and after *in vitro* enzymatic remodeling were separated on 8–16% gradient Tris-glycine

polyacrylamide gels and stained with colloidal blue Coomassie stain. Gels, staining solutions, and molecular weight standards were obtained from Invitrogen (Carlsbad, CA).

Assays of complement regulatory activity

The inhibition of complement-mediated lysis of antibody-sensitized sheep erythrocytes (classical pathway) was assessed as previously described (Scesney *et al.*, 1996).

E-selectin binding assay

E-selectin binding assays were performed according to previously reported methods (Weitz-Schmidt *et al.*, 1996). Flat-bottom 96-well microtiter plates were coated with 5 µg/ml recombinant human E-selectin (R&D Systems) in 150 mM NaCl, 1 mM CaCl₂, 20 mM HEPES, pH 7.4 (HEPES-buffered saline, HBS). Coated wells were blocked with 2% bovine serum albumin/HBS. Varying concentrations of sCR1 or sCR1-sLe^x were added to the plate. A complex of a biotinylated polyacrylamide polymer containing sLe^x (PAA-sLe^x, GlycoTech) and SA-HRP was prepared. A dilution of this conjugate complex was added to the wells containing sCR1 or sCR1-sLe^x or buffer and incubated for 90 min at room temperature. The wells were washed with HBS/CaCl₂ and 3,3',5,5'-tetramethylbenzidine substrate (KPL) was added to each well. Color was allowed to develop for 15 min, and the reaction was stopped with 2.0 N H₂SO₄. Bound PAA-sLe^x complex was measured by determining the absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Pharmacokinetic analysis in rats

Male Sprague-Dawley rats (~250 g), with in-dwelling jugular vein cannulas were purchased from Taconic (Germantown, NY) or Harlan Sprague Dawley (Indianapolis, IN). The catheters were periodically flushed with 0.9% saline followed by either heparinized glycerol (1:4 glycerol/333 IU heparin/ml) or heparinized saline (333 IU/ml) to ensure patency.

Animals were injected with sCR1 or sCR1-S (10 mg/kg) via the lateral tail vein as a bolus at time 0. Blood samples were obtained at timed intervals from the jugular vein cannula. The levels of sCR1 and sCR1-S present in the plasma samples were measured by a previously described enzyme-linked immunosorbent assay (Rittershaus *et al.*, 1999). Briefly, microtiter plates were coated with anti-sCR1 monoclonal antibody 6B1.H12 and captured sCR1 from a sample was detected with an HRP-conjugated anti-sCR1 monoclonal antibody 4D6.1. Pharmacokinetic data was analyzed using WinNonlin (Pharsight, Mountain View, CA).

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Abbreviations

2-AA, 2-antranilic acid; AUC, area under the curve; CHO, Chinese hamster ovary; FACE, fluorophore-assisted

carbohydrate electrophoresis; HBS, HEPES-buffered saline; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-(N-morpholino)ethanesulfonic acid; RP-HPLC, reversed phase high-pressure liquid chromatography; SA-HRP, streptavidin-horseradish peroxidase; sCR1, soluble recombinant complement receptor type 1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

References

- Anumula, K.R. (1994) Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. *Anal. Biochem.*, 220, 275-283.
- Anumula, K.R. (1995) Rapid quantitative determination of sialic acids in glycoproteins by high-performance liquid chromatography with a sensitive fluorescence detection. *Anal. Biochem.*, 230, 24-30.
- Anumula, K.R. and Dhume, S.T. (1998) High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. *Glycobiology*, 8, 685-694.
- Costache, M., Apoil, P.A., Cailleau, A., Elmgren, A., Larson, G., Henry, S., Blancher, A., Iordachescu, D., Oriol, R., and Mollicone, R. (1997) Evolution of fucosyltransferase genes in vertebrates. *J. Biol. Chem.*, 272, 29721-29728.
- Fischer, B.E. and Dorner, F. (1998) Recombinant coagulation factor IX: glycosylation analysis and *in vitro* conversion into human-like sialylation pattern. *Thromb. Res.*, 89, 147-150.
- Goochee, C.F., Gramer, M.J., Andersen, D.C., Bahr, J.B., and Rasmussen, J.R. (1991) The oligosaccharides of glycoproteins: bioprocess factors affecting oligosaccharide structure and their effect on glycoprotein properties. *Biotechnology*, 9, 1347-1355.
- Huang, J., Kim, L.J., Mealey, R., Marsh, H.C. Jr., Zhang, Y., Tenner, A.J., Connolly, E.S. Jr., and Pinsky, D.J. (1999) Neuronal protection in stroke by an sLex-glycosylated complement inhibitory protein. *Science*, 285, 595-599.
- Jenkins, N., Parekh, R.B., and James, D.C. (1996) Getting the glycosylation right: implications for the biotechnology industry. *Nat. Biotechnol.*, 14, 975-981.
- Kallio, E.A., Lemstrom, K.B., Hayry, P.J., Ryan, U.S., and Koskinen, P.K. (2000) Blockade of complement inhibits obliterative bronchiolitis in rat tracheal allografts. *Am. J. Respir. Crit. Care Med.*, 161, 1332-1339.
- Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001a) Moderation of skeletal muscle reperfusion injury by a sLe(x)-glycosylated complement inhibitory protein. *Am. J. Physiol. Cell Physiol.*, 281, C224-C230.
- Kyriakides, C., Wang, Y., Austen, W.G. Jr., Favuzza, J., Kobzik, L., Moore, F.D. Jr., and Hechtman, H.B. (2001b) Sialyl Lewis(x) hybridized complement receptor type 1 moderates acid aspiration injury. *Am. J. Physiol. Lung Cell Mol. Physiol.*, 281, L1494-L1499.
- Lasky, L.A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu. Rev. Biochem.*, 64, 113-139.
- Lazar, H.L., Bao, Y., Gaudiani, J., Rivers, S., and Marsh, H. (1999) Total complement inhibition: an effective strategy to limit ischemic injury during coronary revascularization on cardiopulmonary bypass. *Circulation*, 100, 1438-1442.
- Lee, J., Sundaram, S., Shaper, N.L., Raju, T.S., and Stanley, P. (2001) Chinese hamster ovary (CHO) cells may express six beta 4-galactosyltransferases (beta 4GalTs). Consequences of the loss of functional beta 4GalT-1, beta 4GalT-6, or both in CHO glycosylation mutants. *J. Biol. Chem.*, 276, 13924-13934.
- Lehmann, T.G., Koeppel, T.A., Kirschfink, M., Gebhard, M.M., Herfarth, C., Otto, G., and Post, S. (1998) Complement inhibition by soluble complement receptor type 1 improves microcirculation after rat liver transplantation. *Transplantation*, 66, 717-722.
- Mulligan, M.S., Warner, R.L., Rittershaus, C.W., Thomas, L.J., Ryan, U.S., Foreman, K.E., Crouch, L.D., Till, G.O., and Ward, P.A. (1999)

- Endothelial targeting and enhanced antiinflammatory effects of complement inhibitors possessing sialyl Lewis x moieties. *J. Immunol.*, 162, 4952-4959.
- Naka, Y., Marsh, H.C., Scesney, S.M., Oz, M.C., and Pinsky, D.J. (1997) Complement activation as a cause for primary graft failure in an isogenic rat model of hypothermic lung preservation and transplantation. *Transplantation*, 64, 1248-1255.
- Nemansky, M., De Leeuw, R., Wijnands, R.A., and Van Der Eijnden, D.H. (1995) Enzymic remodelling of the N- and O-linked carbohydrate chains of human chorionic gonadotropin. *Eur. J. Biochem.*, 227, 880-888.
- Nickells, M., Hauhart, R., Krych, M., Subramanian, V.B., Geoghegan-Barek, K., Marsh, H.C. Jr., and Atkinson, J.P. (1998) Mapping epitopes for 20 monoclonal antibodies to CR1. *Clin. Exp. Immunol.*, 112, 27-33.
- Paulson, J.C., Hill, R.L., Tanabe, T., and Ashwell, G. (1977) Reactivation of asialo-rabbit liver binding protein by resialylation with β -D-galactoside α 2-6 sialyltransferase. *J. Biol. Chem.*, 252, 8624-8628.
- Picard, M.D., Pettey, C.L., Marsh, H.C., and Thomas, L.J. (2000) Characterization of N-linked oligosaccharides bearing sialyl Lewis x moieties on an alternatively glycosylated form of soluble complement receptor type 1 (sCR1). *Biotechnol. Appl. Biochem.*, 31(1), 5-13.
- Pratt, J.R., Hibbs, M.J., Laver, A.J., Smith, R.A., and Sacks, S.H. (1996) Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat. *Am. J. Pathol.*, 149, 2055-2066.
- Pruitt, S.K., Bollinger, R.R., Collins, B.H., Marsh, H.C., Levin, J.L., Rudolph, A.R., Baldwin, W.M., and Sanfilippo, F. (1997) Effect of continuous complement inhibition using soluble complement receptor type 1 on survival of pig-to-primate cardiac xenografts. *Transplantation*, 63, 900-902.
- Raju, T.S., Briggs, J.B., Chamow, S.M., Winkler, M.E., and Jones, A.J.S. (2001) Glycoengineering of therapeutic glycoproteins: *in vitro* galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. *Biochemistry*, 40, 8868-8876.
- Rittershaus, C.W., Thomas, L.J., Miller, D.P., Picard, M.D., Geoghegan-Barek, K.M., Scesney, S.M., Henry, L.D., Sen, A.C., Bertino, A.M., Hannig, G., and others. (1999) Recombinant glycoproteins that inhibit complement activation and also bind the selectin adhesion molecules. *J. Biol. Chem.*, 274, 11237-11244.
- Scesney, S.M., Makrides, S.C., Gosselin, M.L., Ford, P.L., Andrews, B.M., Hayman, E.G., and Marsh, H.C. (1996) A soluble deletion mutant of the human complement receptor type 1, which lacks the C4b binding site, is a selective inhibitor of the alternative complement pathway. *Eur. J. Immunol.*, 26, 1729-1735.
- Schmid, R.A., Hillinger, S., Hamacher, J., and Stammberger, U. (2001) TP20 is superior to TP10 in reducing ischemia/reperfusion injury in rat lung grafts. *Transplant. Proc.*, 33, 948-949.
- Shames, S.L., Simon, E.S., Christopher, C.W., Schmid, W., Whitesides, G.M. and Yang, L.L. (1991) CMP-N-acetylneuraminic acid synthetase of *Escherichia coli*: high level expression, purification and use in the enzymatic synthesis of CMP-N-acetylneuraminic acid and CMP-neuraminic acid derivatives. *Glycobiology*, 1, 187-191.
- Stammberger, U., Hamacher, J., Hillinger, S., and Schmid, R.A. (2000) sCR1sLe^x ameliorates ischemia/reperfusion injury in experimental lung transplantation. *J. Thorac. Cardiovasc. Surg.*, 120, 1078-1084.
- Stockert, R.J. (1995) The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol. Rev.*, 75, 591-609.
- Thotakura, N.R., Szkudlinski, M.W., and Weintraub, B.D. (1994) Structure-function studies of oligosaccharides of recombinant human thyroptrophin by sequential deglycosylation and resialylation. *Glycobiology*, 4, 525-533.
- Ward, M. and Power, S.D. (2003) DNA sequences, vectors, and fusion polypeptides for secretion of polypeptides in filamentous fungi. US Patent no. 6,590,078 B2.
- Weisman, H.F., Bartow, T., Leppo, M.K., Marsh, H.C., Carson, G.R., Concino, M.F., Boyle, M.P., Roux, K.H., Weisfeldt, M.L., and Fearon, D.T. (1990) Soluble human complement receptor type 1: *in vivo* inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science*, 249, 146-151.
- Weitz-Schmidt, G., Stokmaier, D., Scheel, G., Nifant'ev, N.E., Tuzikov, A.B., and Bovin, N.V. (1996) An E-selectin binding assay based on a polyacrylamide-type glycoconjugate. *Anal. Biochem.*, 238, 184-190.
- Weston, B.W., Smith, P.L., Kelly, R.J., and Lowe, J.B. (1992) Molecular cloning of a fourth member of a human α (1,3)fucosyltransferase gene family. Multiple homologous sequences that determine expression of the Lewis x, sialyl Lewis x, and difucosyl sialyl Lewis x epitopes. *J. Biol. Chem.*, 267, 24575-24584.
- Williams, J.P., Pechet, T.T., Weiser, M.R., Reid, R., Kobzik, L., Moore, F.D. Jr., Carroll, M.C., and Hechtman, H.B. (1999) Intestinal reperfusion injury is mediated by IgM and complement. *J. Appl. Physiol.*, 86, 938-942.
- Zacharowski, K., Otto, M., Hafner, G., Marsh, H.C. Jr., and Thiemermann, C. (1999) Reduction of myocardial infarct size with sCR1sLe(x), an alternatively glycosylated form of human soluble complement receptor type 1 (sCR1), possessing sialyl Lewis x. *Br. J. Pharmacol.*, 128, 945-952.
- Zhang, A., Potvin, B., Zaiman, A., Chen, W., Kumar, R., Phillips, L., and Stanley, P. (1999) The gain-of-function Chinese hamster ovary mutant LEC11B expresses one of two Chinese hamster FUT6 genes due to the loss of a negative regulatory factor. *J. Biol. Chem.*, 274, 10439-10450.

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